University of Alberta

Intestinal Sugar Transport: From Early Development to Senescence

by

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requirements for the degree of Doctor of Philosophy

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ABSTRACT

Sugars represent an important caloric component of the diet of mammals. The rate of sugar uptake falls in early life, and may fall further with aging. In mature animals, sugar uptake may be increased by glucagon-like peptide 2 (GLP-2) and dexamethasone (DEX) and may be increased be feeding a diet enriched with saturated fatty acids (SFA) when compared to a diet enriched in polyunsaturated fatty acids (PUFA). Accordingly, this thesis was undertaken to test five hypotheses: 1) there is a continuum of decline in intestinal sugar uptake that occurs throughout the lifespan of the animal: 2) the normal decline in intestinal sugar uptake may be prevented by administering GLP-2 and DEX early in life; 3) feeding a diet enriched in SFA as compared to PUFA increases intestinal sugar uptake later in life; 4) the changes in intestinal sugar uptake with age, with GLP-2 and DEX, and with variations in dietary lipids, are associated with parallel alterations in intestinal morphology and transporter protein abundance; and 5) the changes in intestinal sugar uptake with age, GLP-2 and DEX, and variations in dietary lipids, are associated with directionally similar alterations in selected signalling proteins. The results confirm that 1) there is a progressive decline, followed by a plateau in intestinal sugar uptake over the lifetime of the animal; 2) the normal decline in sugar uptake in early life can be prevented by GLP-2 or DEX; and 3) the uptake of sugars in older rats is enhanced by feeding a diet enriched in PUFA. The data also demonstrate that 1) the changes in sugar uptake associated with altering dietary lipid composition are associated with alterations in intestinal morphology; 2) the age-and hormone-associated changes in sugar uptake

are not associated with parallel alterations in intestinal morphology or transporter abundance; 3) however, these changes are associated with directionally similar alterations in the abundance of signalling proteins that are members of the PI3-kinase signalling pathway. Thus, we speculate that the age- and hormone-associated changes in sugar uptake are due to alterations in the intrinsic activity of the sugar transporters signalled by the PI3-kinase signalling pathway.

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LIST OF ABBREVIATIONS

3-OMG	3-*O*-methylglucose
11β-HSD	11β-hydroxysteroid dehydrogenase
ACTH	adrenocorticotropin hormone
Akt/PKB	Protein kinase B
AMPK	AMP-activated kinase
ATP	adenosine triphosphate
BBM	brush border membrane
BLM	basolateral membrane
BLOTTO	Bovine Lacto Transfer Technique Optimizer
CBG	corticosteroid binding globulin
C/EBP	CCAAT/enhancer binding proteins
CREB	cAMP response element binding protein
D-19575	beta-D-glucosylisophosphoramide
DAB	diaminobenzidine
DEX	dexamethasone
DHEA	dehydroepiandrosterone
DOG	*sn*-1,2-dioctanoylglycerol
DPPIV	dipeptidyl peptidase IV
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
Epo	erythropoietin
ER	endoplasmic reticulum
ERG	early response genes
ERK1/2	extracellular regulated kinase 1/2
F	fructose
FAT/CD36	fatty acid transporter
FBS	Fanconi Bickel syndrome
G6PT	glucose-6-phosphate translocase

G6PT1	glucose-6-phosphate translocase
GC	glucocorticosteroids
GGM	glucose-galactose malabsorption
GH	growth hormone
Glc	glucose
Glc-6-P	glucose-6-phosphate
GLP-1	glucagon-like peptide 1
GLP-2	glucagon-like peptide 2
GLP-2R	glucagon-like peptide 2 receptor
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
GLUT4	glucose transporter 4
GLUT5	glucose transporter 5
GLUT7	glucose transporter 7
GPCR	G protein coupled receptor
GR	glucocorticoid receptor
GSK-3	glycogen synthase kinase 3
HGF	Hepatocyte growth factor
HNF-1	hepatic nuclear factor 1
HNF-4	hepatic nuclear factor 4
HPN	home parenteral nutrition
HRP	horseradish peroxidase
HSF1	heat shock transcription factor 1
Hsp70	heat shock protein 70
I-FABP	intestinal fatty acid binding protein
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IL1-β	interleukin 1 βeta
ILBP	ileal lipid binding protein
IRS	insulin receptor substrate
KGF	keratinocyte growth factor

KGFR	keratinocyte growth factor receptor
Km	Michaelis affinity constant
LCM	laser capture microdissection
L-FABP	liver fatty acid binding protein
LPH	lactase phlorizin hydrolase
MAPK	mitogen activated protein kinase
MEK	mitogen-activated kinase kinase
MSBR	massive small bowel resection
MSS	membrane suspension buffer
mTOR	mammalian target of rapamycin
Na^+K^+-ATPase	sodium potassium adenosine triphosphatase
NEC	necrotizing enterocolitis
NFκB	nuclear factor κB
NOS3	nitric oxide synthase 3
ODC	orinithine decarboxylase
OKG	ornithine α-keto glutarate
ORT	oral rehydration therapy
PARP	poly ADP-ribose polymerase
PBR	peripheral benzodiazepine receptor
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDK-1	protein-dependent kinase-1
Pi	phosphate
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

Ponceau S	3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2,7-naphtalenediasulfonic acid
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acids
RS1	regulatory subunit 1
RT-PCR	reverse transcription-polymerase chain reaction
S4048	glucose-6-phosphate translocase inhibitor
SAP	steroidogenesis activator protein polypeptide
SCP2	sterol carrier protein 2
SBS	short bowel syndrome
sc	subcutaneous
SFA	saturated fatty acid
SGLT1	sodium dependent glucose transporter
SI	sucrase-isomaltase
SPARC	secreted protein, acidic and rich in cystine
SREBP1c	sterol response element binding proteins 1c
StaR	steroidogenic acute regulatory protein
T3	triiodothyronine
T4	thyroxine
TGF-β	transforming growth factor-β
TNF-α	Tumor necrosis factor-α
TNF-α	tumor necrosis factor-α
TTBS	Tween tris buffered saline
TUNEL	terminal dUTP nick-end labeling
Vmax	maximal transport rate

1. INTRODUCTION

What information was available to me when I started my thesis on the profile of intestinal sugar uptake over the lifespan of the animal? A collaborative study between our laboratory and researchers at McGill University (Wild et al., 2005, unpublished observations) profiled the mRNA expression and protein abundance during early development in neonatal rats (3-35 days of age). This study showed that glucose transporter (SGLT1, GLUT2) mRNA expression peaked at postnatal day 35, and that the protein abundance of the transporters followed a similar timeline. This study by Wild and colleagues did not measure sugar uptake, but it was speculated that the transporter protein was functional in these young animals. Others have suggested that glucose uptake peaks shortly after birth (postnatal day 3), when the intestine takes over the burden of nutrient acquisition from the placenta (Toloza and Diamond, 1992). Furthermore, kinetic studies done on pigs demonstrate that maximum rates of transport for D-glucose were highest immediately after birth even before suckling, with a subsequent decrease associated with the onset of suckling (Puchal and Buddington, 1992).

Although SGLT1 and GLUT2 are expressed both in the rat fetus and at birth, the expression of brush border membrane (BBM) GLUT5 is delayed, and is only significantly detected in post weaning animals (Castello et al., 1995; Rand et al., 1993; Shu et al., 1997; Toloza and Diamond, 1992). In rats, GLUT5 protein and mRNA abundance parallel fructose transport, and therefore remain low throughout the suckling phase (day 1-14), with higher levels detected in the weaning phase (day 15-27) and post-weaning phase (day 28-42) (Toloza and Diamond, 1992; Rand et al., 1993; Castello et al., 1995). This increase in fructose uptake coincides with the appearance of fructose in the pups' diet, and parallels the increase in fructose uptake seen at this time. Although there is a temporal association between dietary fructose and the appearance of GLUT5 in the BBM, the expression of the transporter is "hard wired" and occurs at this time even in the absence of dietary stimuli (Shu et al., 1998).

There are also changes in intestinal sugar transport in later life, but experiments using rodent models of aging demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985). Depending upon the intestinal site studied, a normal or increased absorptive capacity was also found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption. Ferraris et al. (1993) showed a reduction in uptake and site density of SGLT1 in aged mice. This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice.

The uptake of fructose has been studied in aging mice. Ferraris and Vinnekota (1993) showed that D-fructose uptake per milligram of tissue was higher in the jejunum of young as compared to old animals. Adaptive increases in uptake, in response to increases in dietary carbohydrate levels, were blunted in these mice, and were restricted to the more proximal regions of the small intestine.

Thus, the literature supports the concept that there is an early decrease in intestinal sugar uptake shortly after birth and also suggests a further decline later in life. The changes that occur early in life are not usually considered with the changes that occur in later life and *vice versa*. When I took this perspective and looked at the process that occurs over a lifetime, I developed my first hypothesis:

HYPOTHESIS #1: There is a continuum of decline in intestinal sugar uptake that occurs throughout the lifespan of the healthy rat

The process of intestinal adaptation has been reviewed (Thiesen et al., 2003a). For example, sugar uptake is up-regulated in diabetes (Thomson et al., 1987a), following intestinal resection (Robinson et al., 1982), or by feeding a diet enriched in saturated fatty acids (Thomson et al., 1986; 1987b; 1988). Conversely, intestinal sugar uptake is down-regulated with total parenteral nutrition (Inoue et al., 1993), abdominal radiation (Thomson et al., 1983), and by feeding a diet enriched in polyunsaturated fatty acids as compared with saturated fatty acids (Thomson et al., 1986; 1987b; 1988). There are two main approaches used to physiologically alter intestinal nutrient absorption: 1) vary the amount or type of nutrients in the diet; or 2) administer selected hormones or chemicals known to influence this process. Because our laboratory has already published studies looking at the effect of early dietary changes on intestinal transport (Perin et al., 1997; Jarocka-Cyrta et al., 1998; Perin et al., 1999), we chose to take a different, yet novel and potentially clinically significant approach: We looked at the influence of a glucocorticosteroid (dexamethasone, DEX) and GLP-2 on the ontogeny of intestinal sugar transport.

There is a considerable literature on the effect of steroids on the precocious induction of BBM enzymes (Scott, 1980; Batt and Scott, 1982; Martin and Henning, 1982). Steroids have been previously shown to increase sugar uptake in adult animals (Thiesen et al., 2003b; Thiesen et al., 2003c). However, the influence of steroids on sugar uptake, when given directly to young suckling animals is unknown. Also, it is not known if maternal steroids influence intestinal sugar transport in their offspring, or if these effects persist.

GLP-2 increases intestinal morphology and sugar transport in adult animals (Drucker et al., 1996; Tsai et al., 1997; Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998). Thus, both GLP-2 and DEX stimulate intestinal sugar uptake. The role of GLP-2 in the ontogeny of sugar transport is unknown. Accordingly, we developed our second hypothesis:

HYPOTHESIS #2: The normal decline in intestinal sugar uptake may be prevented by administering GLP-2 and DEX early in life.

There is an extensive literature on the adaptation of the intestine in response to alterations in both the amount and the type of nutrients in the diet. For example, there are increases in intestinal lipid uptake with high fat diets (Sukhotnik et al., 2003), increases in intestinal glucose uptake with high carbohydrate diets (Ferraris et al., 1992; Ferraris and Diamond, 1997), and increases in intestinal fructose uptake with early fructose feeding (Shu et al., 1998). In adult animals, diets enriched with saturated fatty acids (SFA) increase sugar uptake when compared to isocaloric diets enriched in polyunsaturated fatty acids (PUFA) (Thomson et al., 1986; 1987b; 1988).

The effect of aging on intestinal adaptation is unclear. Some investigators have suggested that the ability to adapt is reduced with aging. For example, Ferraris and Vinnekota (1995) showed that the intestinal adaptive increases in response to a high carbohydrate diet were reduced in old animals, and changes were limited to the proximal small intestine. Other studies have also demonstrated that responsiveness to dietary changes is not lost with aging: Following 3 days of starvation, aged animals demonstrated an exaggerated BBM enzyme response (lactase, sucrase, maltase) to refeeding (Holt and Kotler, 1987; Holt et al., 1988).

As previously mentioned, in adult animals SFA increases sugar uptake when compared to PUFA (Thomson et al., 1986; 1987b; 1988). But until I began my studies, it was not known if we could apply this information on intestinal transport adaptation obtained in younger rats to older animals. Therefore, we sought to determine if the aged intestine behaves in a similar fashion as the younger intestine. Accordingly, we developed our third hypothesis:

HYPOTHESIS #3: Feeding a diet enriched with saturated as compared to polyunsaturated fatty acids increases intestinal sugar uptake later in life.

The potential mechanisms by which variations in intestinal sugar uptake may occur are numerous. Non-specific changes may occur via alterations in intestinal morphology or by changes in the ratio of transporting to non-transporting enterocytes (reviewed in Ferraris and Diamond, 1997). Alternatively, specific changes may occur due to alterations in the abundance of transporter protein. Accordingly, we developed our fourth hypothesis:

HYPOTHESIS #4: The changes in intestinal sugar uptake with age, GLP-2 and DEX, and variations in the type of dietary lipids, are associated with parallel alterations in intestinal morphology and transporter abundance.

Furthermore, in some specific models, such as with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000), and following the activation of MAPK and PI3K (Helliwell et al., 2000a), alterations in sugar uptake may also occur as a result of changes in the intrinsic activity of the transporters, in the absence of variations in transporter protein abundance. The signalling pathways involved in modulating transporter activity have not been fully elucidated, although there is evidence that intestinal glucose uptake is modulated by PKC and PKA (Vayro and Silverman, 1999; Helliwell et al., 2000b; Veyhl et al., 2003), as well as the MAPK and PI3K signalling pathway (Helliwell et al., 2000a; Alexander and Carey, 2001; Millar et al., 2002). Accordingly, we developed our fifth hypothesis:

HYPOTHESIS #5: The changes in intestinal sugar uptake with age, GLP-2 and DEX, and variations in the type of dietary lipids, are associated with directionally similar alterations in selected signalling proteins.

The next section of this thesis contains an extensive Literature Review. This shall provide a context in which to further understand the rationale for the hypotheses. The broad range of topics will weave together information from the areas of Intestinal Sugar Transport, Intestinal Adaptation, Ontogeny, Aging and Hormones and Growth Factors.

This thesis is in "paper format". The five major findings are:

- Intestinal sugar uptake decreases with age (Ch. 4,5,10,11)
- GLP-2/DEX influence intestinal sugar uptake (Ch. 8-11)
- SFA decreases intestinal sugar uptake in old animals (Ch. 6,7)
- Changes in uptake are not associated with parallel changes in morphology and transporter abundance (Ch.4-11)
- Changes in uptake are associated with parallel changes in selected signalling proteins(Akt, mTOR) (Ch.4-11)

The data and discussion that is relevant to each of these findings is located in the Chapters indicated above. In the Summarizing Discussion I integrate the experimental findings and propose future studies.

2. LITERATURE REVIEW

2.1 INTESTINAL SUGAR TRANSPORT

It has been known for decades that two different processes existed for intestinal glucose and fructose absorption. In studies using everted sacs of hamster small intestine, Crane and colleagues found that when the serosal and the mucosal side of the tissue were bathed in glucose, glucose accumulated on the serosal side (Wilson and Crane, 1958). This was not the case for fructose, and therefore the absorptive process was labelled as non-concentrating. The involvement of sodium (Na+) in glucose absorption was first proposed by Riklis and Quastel (1958), although studies had previously demonstrated that the decrease in sugar absorption seen in adrenalectomized animals was prevented by adding NaCl to the drinking water (Clark and MacKay, 1942). The original Na^+/glucose cotransport hypothesis was presented by Crane in the 1960's (Crane et al., 1962). This group showed that active glucose absorption by hamster small intestine required sodium (Na^+) in the bathing medium. Glucose transport was also blocked by ouabain which inhibits the Na^+K^+-ATPase in the basolateral membrane (BLM). This protein is responsible for maintaining the Na^+ gradient in the enterocytes, and driving Na^+dependent transporters such as the sodium-dependent glucose transporter (SGLT1) in the brush border membrane (BBM).

Crane further developed the model of a mobile carrier in the BBM with two binding sites, one for glucose and one for Na^+ (Crane, 1965). He determined that the continuously maintained outward Na^+ gradient accomplished by the Na^+K^+-ATPase on the BLM was the primary asymmetry providing the driving force for active sugar transport. The phenomenon was considered to be "secondary active transport", as the hydrolysis of ATP was indirectly coupled to glucose transport via this electrochemical gradient. This pioneering work provided the framework for the further characterization of not only glucose transport, but also the transport of other co-transported solutes, and this concept is now considered to be a central tenet in cell physiology.

The pioneering work done by Crane was followed by the electrophysiological studies of Curran and colleagues (Curran, 1960; Curran, 1965; Schultz and Curran, 1970) that further characterized transcellular Na^+ transport, and increased the understanding of Na^+ coupled co-transport. Further important advances were made in the 1980's. The method of expression cloning, developed by Wright and colleagues, resulted in SGLT1 being the first eukaryote cotransporter to be cloned. This technique takes advantage of the fact that Xenopus oocytes have the unique ability to translate foreign mRNA, and insert functional transporters into their plasma membrane. The researchers injected rabbit polyA RNA into the oocytes, and observed increases in glucose transport. Utilizing molecular techniques, they were able to isolate a single clone, and use it as a probe to identify human SGLT1 (Hediger et al., 1987).

With the continuing development of molecular techniques, the process of intestinal sugar absorption was developed further. The cloning and characterization of the sugar transporters GLUT2 (Thorens et al., 1988) and GLUT5 (Burant et al., 1992) soon followed, and the molecular aspects of the process of sugar absorption across the BBM and BLM were characterized. What is now known as the *"classical model of sugar absorption"* was developed (Figure 1), with SGLT1 actively transporting glucose and galactose across the BBM, and fructose crossing the BBM by facilitative diffusion via GLUT5. GLUT2, a low affinity transporter, was responsible for transporting these sugars across the BLM via facilitative diffusion.

SGLT1

Hediger et al. (1987) cloned the SGLT1 gene. The cotransporter is a 73 kDa membrane protein with a Na^+-glucose stoichiometry of 2:1. The transporter has the same affinity for both glucose and galactose (Table 1), and transport is phloridzin sensitive (Ki=0.1 uM) (Table 2). The membrane topology of SGLT1 was determined using N-glycosylation scanning mutants and hydropathy plots. The transporter contains 14 transmembrane alpha-helices, with an extracellular N and C terminus (Turk and Wright, 1997; Panayotova-Heiermann et al., 1997; Turk et al., 1996). The transporter contains a single glycosylation site between transmembrane 5 and 6; however, glycosylation is not required for functioning of the protein. Phosphorylation sites have been identified between transmembrane helices 6 and 7, and between transmembrane helices 8 and 9 (Wright et al., 1992). The importance of SGLT1 phosphorylation will be discussed below. SGLT1 is found in the small intestine, with very small amounts detectable in the kidneys and the heart. Recently, SGLT1 has also been detected in the luminal membrane of intracerebral capillary endothelial cells, where it may participate in the transport of glucose across the blood-brain barrier (Elfeber et al., 2004).

The process of intestinal sugar transport has been reviewed by Wright et al. (2003). Initially, on the luminal side of the BBM, two Na^+ ions bind to SGLT1 and produce a conformational change that permits sugar binding. Another conformational change allows the substrates to enter the enterocyte. The sugar, followed by the Na^+, dissociates from SGLT1 because the affinity of the cytosolic sites is low, and also because the intracellular concentration of Na^+ is low (10 vs. 140 mEq/L). Sodium can be replaced by H^+ or Li^+, but the affinity for glucose then decreases (apparent Michaelis affinity constant (Km) = 4-11 mM).

The Na^+K^+-ATPase in the BLM is responsible for maintaining the Na^+ and K^+ electrochemical gradients across the cell membrane. The Na^+K^+-ATPase contains a 110 kDa α_1 catalytic subunit, as well as a highly glycosylated 55 kDa β_1 subunit (Fambrough et al., 1994; Horisberger et al., 1991). The Na^+K^+-ATPase is up-regulated in experimental diabetes (Wild et al., 1999) and experimental ileitis (Wild and Thomson, 1995), with post-translational modifications playing an important role in its regulation.

Panayotova-Heiermann and Wright (2001) expressed various cDNA constructs of rabbit SGLT1 in Xenopus oocytes in order to determine the helices involved in sugar transport. They found that helices 10-13 form the sugar permeation pathway for SGLT1, and they speculated that the N terminal region of SGLT1 (helices 1-9) may be required to couple Na^+ and glucose transport.

A number of factors influence the transport function of SGLT1 (Table 3). For example, the regulation of SGLT1 by dietary sugars was examined by Miyamoto et al. (1993). Using Northern blotting, they showed that SGLT1 mRNA was increased by feeding rats 55% sugar diets containing glucose, galactose, fructose, mannose, xylose, or 3-O-methylglucose. Because 3-O-methylglucose is transported by SGLT1, but is not metabolized, and because SGLT1 does not transport fructose, mannose or xylose, the up-regulation of SGLT1 does not appear to depend on either metabolism or transport of the sugar in question (Table 3).

Wright et al. (1997) evaluated the role of SGLT1 phosphorylation. They expressed rabbit SGLT1 in Xenopus oocytes, and activated protein kinase A (PKA) or protein kinase C (PKC) using 8-Br-cAMP and *sn*-1,2-dioctanoylglycerol (DOG), respectively. PKA activation increased glucose transport by approximately 30%, while PKC activation reduced transport by 60%. The change in maximal transport rates (Vmax) was accompanied by alterations in the number of transporters in the plasma membrane, as well as changes in the surface area of the membrane. Since endocytosis and exocytosis alter the membrane surface area, the findings of the effects of PKA and PKC on SGLT1 suggest that these proteins may be involved in the regulation of glucose transport.

Similar increases in Vmax were obtained with activation of PKA in oocytes expressing rabbit, human, and rat SGLT1 isoforms. The effects of PKC, however, may depend on the sequence of the co-transporter, as there are conflicting reports of the effect of PKC on glucose transport. For example, PKC decreases sugar transport in Xenopus oocytes expressing rabbit and rat SGLT1 (Vayro et al., 1999; Vehyl et al., 2003), and increases sugar transport when human SGLT1 is expressed (Hirsch et al., 1996).

Veyhl et al. (1993) demonstrated the presence of an intracellular regulatory protein (RS1) that may modify the activity of SGLT1. Co-expression of RS1 and SGLT1 in Xenopus oocytes reduced both the Vmax for glucose transport as well as SGLT1 protein levels (Veyhl et al., 2003). Plasma membrane surface area was also reduced, suggesting endo- and exo-cytosis of membrane vesicles.

To investigate the role of intracellular trafficking in sugar transport, oocytes were injected either with cRNA of wild type, or mutant dynamin. Dynamin is a protein involved in receptor-mediated endocytosis, vesicle recycling, caveolae internalization and vesicle trafficking from the Golgi (Hinshaw, 2000). The inhibition of glucose uptake by RS1 was largely reduced after co-expression of the mutant dynamin protein. The investigators concluded that RS1 modulates dynamin-dependent trafficking to the BBM of intracellular vesicles containing SGLT1.

In order to further characterize the role of the RS1 protein in the regulation of intestinal glucose transport, a knockout mouse lacking the RS1 protein was recently developed: Osswald et al. (2005) showed that RS1-/- mice developed obesity associated with increases in food intake, glucose transport and SGLT1 expression in the small intestine. The effect of RS1 deficiency was tissue-specific and occurred through post-transcriptional mechanisms, as SGLT1 mRNA abundance was unchanged. These researchers speculated that therapeutic strategies aimed at reducing glucose uptake by increasing RS1 may potentially be used to treat obesity.

Heat shock proteins (hsp) may also play a role in regulating SGLT1 function (Table 3). A study done in renal epithelial cells showed that treatment with hsp70 increased glucose transport, but not the abundance of SGLT1 protein (Ikari et al., 2002). The increase in sugar transport was inhibited by an antibody directed against transforming growth factor β (TGF- β), leading the investigators to explore the effect of TGF-β on SGLT1: there was an increase in SGLT1 activity, as well as an increase in hsp70 protein when TGF-β was added to the culture media. The researchers speculated that hsp70 may stabilize SGLT1 expression in the membrane. This concept was supported by confocal microscopy studies, demonstrating that SGLT1 and hsp70 co-localized to the BBM.

It is not known if SGLT1 is localized to specific microdomains within the BBM. In renal proximal tubular cells, SGLT1 was found in detergent-resistant membrane microdomains, also referred to as "lipid rafts" (Runembert et al., 2002). In this model, the absence of vimentin, an intermediate filament component, decreased glucose transport and caused a reduction in the amount of SGLT1 protein in these membrane microdomains. Furthermore, fluidization of the plasma membrane, or depleting the membrane of cholesterol, dramatically decreased glucose transport. This suggests that the activity of SGLT1 is optimal in a microenvironment characterized by low fluidity. Further research is required to determine if SGLT1 is localized to lipid rafts in the intestinal BBM, if this localization is mandatory for the functioning of SGLT1, and what are the factors that may regulate the localization of SGLT1 to these specialized microdomains.

The transcription factors hepatocyte nuclear factor-1 (HNF-1) and Sp1 may also regulate SGLT1. Martin et al. (2000) characterized the promoter region of the SGLT1 gene by transfecting reporter constructs into Caco-2 cells. They demonstrated that three cis-elements, HNF-1, and two sites on the SGLT1 promoter ("GC boxes" to which Sp1 binds are required for maintaining basal transcription of SGLT1. Members of the Sp1 family bind to the GC boxes, and in the presence of HNF-1, synergistically activate the promoter. Some members of the Sp1 family have been implicated in tissue- and developmental- specific regulation of genes (Lania et al., 1997; Saffer et al., 1991). HNF-1 alters the expression of many small intestinal genes, including sucrase-isomaltase (SI) and lactase. It has also been implicated in the diurnal regulation of SGLT1 in rodents (Rhoads et al., 1998). If HNF-1 was required for basal SGLT1 expression, glucose-galactose malabsorption would have been expected to be observed. Of interest, HNF-1 knockout mice experience life-threatening effects on the hepatic and renal systems, but no adverse effects on the gastrointestinal tracts were reported ..

Katz et al. (2004) identified a link between a mesenchymal factor and the regulation of a specific epithelial transport process. *Foxl1* is a winged-helix transcription factor expressed in the mesenchymal cells bordering the crypts in the small intestine. Using the everted sleeve method coupled with Western blotting, the researchers showed that homozygous *Foxl1* null mice had decreased intestinal glucose uptake and decreased levels of SGLT1 protein. Growth retardation and abnormal small intestinal architecture were observed, characterized by short, broad and irregular villi. The effect of the loss of Foxl1 on SGLT1 was specific, as no changes in the expression of SI, lactase, GLUT2 or Na^+K^+ATPase were observed.

The transport of water across the intestinal epithelia has always been a subject of curiosity. The discovery of aquaporins by Preston et al. (1992) renewed interest in this topic. Although aquaporins may account for a portion of water absorption in the intestine, Wright and colleagues investigated the coupling of water transport to active Na^+-glucose cotransport. Overexpression of human or rabbit SGLT1 in Xenopus oocytes revealed that activation of the transporter was associated with an increase in volume of the cell (reflecting water transport), and this effect was blocked by phlorizin. If oocytes expressing SGLT1 were incubated in a sugar-free solution, no change in oocyte volume was observed. The increase in volume could be accounted for by a stoichiometry of two Na+ ions, one glucose molecule, and 249 water molecules (Loo et al., 2002). The transport of water was independent of the osmotic gradient across the membrane, and may be a consequence of the conformational changes in SGLT1 that occur during Na^+/glucose transport. A channel formed by five C-terminal transmembrane helices of SGLT1 is thought to transport not only water, but also urea (Leung et al., 2000; Panayotova-Heiermann and Wright, 2001).

Oral rehydration therapy (ORT) was developed in the 1970's to treat diarrheal dehydration (Hirschhorn et al. 1973). The introduction of this very simple treatment has reduced mortality due to diarrhea in children under five years of age from 5 million in 1978, to 1.3 million in 2002 (http://www.who.int/child-adolescent-health.2002; Victora et al., 2000). This success led to the proclamation that ORT was the "most important medical advance of the 20th century", and earned Dr Hirschhorn and colleagues the first Pollin prize for Pediatric Research.

The goals of ORT are to replace fluids and minimize malnutrition. Starting in 1978, solutions containing a mixture of glucose, sodium, chloride, potassium and citrate were being commonly distributed by the World Health Organization. In fact, 800 million packets of ORT were distributed worldwide in 1991-1992 (Victora et al., 2000). Interestingly, controversy now exists over the optimal formulation, with reduced osmolarity formulas, rice-based formulas, or formulas containing amylase-resistant starch being favored by some researchers. For example, hypoosmolar rice-based formulas produced better results in cholera patients when compared to standard formulas (Dutta et al., 2000). The advantages of this rice-based formula is that it is cheap, offers more calories than standard ORT, and rice is readily available in many cholera-stricken regions. ORT formulas containing amylase-resistant starches may be favored due to the production of short chain fatty acids, which increase colonic Na+, Cl- and fluid absorption, and reduce colonic secretions (Sellin et al., 1999; Binder and

Mehta, 1989; Resta-Lenert et al., 2001). These effects counteract the fluid losses and hypersecretion seen with infectious diarrhea.

Several features of carbohydrate digestion contribute to the efficacy of ORT. This life-saving therapy is based on the ability of SGLT1 to co-transport water. Na^+-dependent glucose absorption is not affected by the increased cAMP levels commonly seen with infections such as Cholera, and therefore this physiological fact can be exploited as a means to achieve glucose, Na^+ and water absorption, even in the presence of chloride and water secretion. Also, the oral administration of glucose or carbohydrates up-regulates SGLT1, thereby further increasing the intestinal transport of glucose, Na^+ and water. Since ORT is commonly administered to infants, it is important to utilize a transport system that is expressed and functional early in life. SGLT1 is expressed prenatally (Rubin, 1992), and is functional at birth, making it an ideal candidate. In contrast to glucose, the use of fructose in these ORT solutions is contraindicated, as GLUT5 in the BBM is only expressed following weaning (Rand et al., 1993).

Exploiting SGLTs for other purposes is currently under investigation. For example, cancerous cells from various tissues may demonstrate increases in glucose uptake, coupled with an over-expression of members of the GLUT and SGLT families (Younes et al., 1996; Younes et al., 1997; Zamora-Leon et al., 1996). Veyhl et al. (1998) characterized the transport of the chemotherapeutic agent D-19575 (beta-D-glucosylisophosphoramide) in Xenopus oocytes expressing cloned Na^+-glucose transporters. They determined that the drug was transported in a phlorizin-inhibitable manner and was accumulated in the cell. They suggested that SGLT3 (another member of the SGLT family of transporters which is thought to act as a glucose sensor), transported this drug. This demonstrates the potential for the glucose transport system to deliver drugs to cancer cells.

Agents that induce a hypoglycaemic effect via direct or indirect actions on glucose transport proteins have a potential use in the treatment of diabetes (reviewed in Asano et al., 2004). For example, agents that increase GLUT4 trafficking in muscle and adipose cells, thereby reducing blood glucose concentrations, may be potential anti-diabetic drugs. The use of the SGLT1 inhibitor phloridzin as an anti-diabetic agent is limited due to its low oral bioavailability. However, a derivative of phloridzin, T-1095, is absorbed into the circulation and metabolized to an active form. T-1095 lowers blood glucose concentrations, mainly by increasing glucose excretion in the urine, and may have therapeutic value in treating hyperglycemia (Oku et al., 1999).

Glucose-galactose malabsorption (GGM) is a very rare autosomal recessive disease characterized by severe life-threatening diarrhea in the neonate, that resolves when the offending sugars (glucose, galactose and lactose) are removed from the diet (Wright et al., 2002). Normal intestinal mucosal histology is observed, while phlorizin binding studies show reductions in SGLT1 protein in the BBM (Schneider et al., 1966; Stirling et al., 1972). Electrophysiological studies and freeze fracture electron microscopy showed that this disease is due to a failure of the SGLT1 protein to traffic normally to the BBM (Martin et al., 1997). Approximately 300 cases of GGM have been identified worldwide, affecting all racial and ethnic groups. The majority (70%) of patients are female, with two thirds coming from a consanguineous relationship (Wright et al., 2003). Unlike genetic diseases like cystic fibrosis, in which a single mutation accounts for most cases, in GGM each patient appears to have a unique mutation, ranging from missense mutations, to frame-shift mutations, to split-site- conservative mutations which produce truncated protein and mistrafficking of SGLT1 to the BBM (Turk et al., 1991; Martin et al., 1996; Martin et al., 1997; Lam et al., 1999). This variety of mutations limits the usefulness of genetic testing for GGM, although prenatal diagnosis in a family at risk may be possible.

GGM is a difficult condition to diagnose. If GGM is suspected, the first step is the elimination of glucose, galactose and lactose from the infant's diet. Oral glucose tolerance tests in GGM patients produce a flat glucose response in the blood, as glucose is malabsorbed in the intestine. A hydrogen breath test performed following oral glucose produces abnormally high concentrations of H_2 in the breath (> 20 ppm) indicating glucose malabsorption, while oral fructose tolerance tests produce normal results. GGM is treated by using glucose-, galactose- and lactose-free formulas, and by eliminating the offending sugars from the diet (Wright et al., 2003). Normal growth and neurological development are possible if infants receive fructose-based formulas, and if dietary counselling is available (Abad-Sinden et al., 1997; Wright, 1998).

GLUT5

GLUT5 is a 43 kDa protein, with 12 transmembrane domains and intracellular N and C terminals. It was cloned by Burant and colleagues in 1992. GLUT5 was expressed in Xenopus oocytes, and its substrate specificity and kinetic properties were determined using radiolabelled substrates. Northern and Western blotting demonstrated the presence of GLUT5 in human small intestine and testis. Further work by Davidson et al. (1992) focused on the developmental expression of GLUT5 in the human and fetal small intestine. GLUT5 mRNA levels increase with age, and are highest in the adult small intestine. In adults, GLUT5 was localized to the BBM by Western blotting. Immunohistochemical techniques confirmed this finding, and further localized GLUT5 to only the mature enterocytes populating the upper half of the villus. This luminal localization provided further support for the notion that GLUT5 played a role in the intestinal uptake of dietary sugars.

Rand et al. (1993) characterized the expression of GLUT5 in rats. A distinct pattern of expression was seen along the crypt-villous axis, with GLUT5 mRNA being highest in midvillus region. A proximal-distal gradient was also observed, with GLUT5 mRNA levels being higher in the proximal small intestine when compared to the distal small intestine. GLUT5 mRNA was detected in the small intestine, kidney and brain by Northern blotting, and in the small intestine, testis, adipose and skeletal muscle using *in situ* hybridization.

The functional domain of GLUT5 was investigated by Buchs et al. (1998). In order to ensure proper transport and insertion into the membrane, GLUT5-GLUT3 chimeras were created, and included various combinations of the GLUT3 and GLUT5 peptides. These chimeric GLUTs were expressed in Xenopus oocytes. This enabled the researchers to conclude that the regions necessary for fructose transport lie between the amino terminus and the third transmembrane domain, and between the 5th and 11th transmembrane domain.

The response of GLUT5 to dietary sugars was investigated by Miyamoto et al. (1993). In this study, they fed sugar-enriched diets (55% D-glucose, D-galactose, 3-0-

methylglucose, D-fructose, D-mannose or D-xylose) to male Sprague Dawley rats for 5 days. Northern blotting on intestinal samples showed that GLUT5 mRNA was increased only by dietary D-fructose, and was unaffected by the other sugars (Table 3). This was consistent with the suggestion that GLUT5 was a high affinity fructose transporter. Subsequent work by David et al. (1995) showed that in 16 day old rats, feeding fructose but not glucose increased fructose uptake. Furthermore, while both fructose and sucrose feeding enhanced absorption in older (21-60 day old) animals, glucose alone had no effect.

An interesting study by Castello et al. (1995) demonstrated that GLUT5 mRNA in rats followed a circadean rhythm, with a 12-fold increase in mRNA at the end of the light cycle as compared to early in the light cycle. BBM GLUT5 protein followed a similar pattern, which is also observed for other small intestinal genes such as BBM SI and lactase (Saito et al., 1980). Although this pattern was thought to be a reflection of rodent feeding patterns, Corpe et al. (1996) found that gene expression is hard-wired, because GLUT5 is up-regulated prior to the onset of feeding, even in the absence of dietary fructose. Shu et al. (1998) noted that this circadean rhythm was not developed at the time of weaning, possibly because the feeding patterns of suckling rats do not follow the same adult nocturnal patterns. This diurnal variation in adult animals needs to be carefully considered when designing experiments in which levels of GLUT5 are measured, by performing studies in the morning in the early post-prandial period.

The regulation of GLUT5 was studied by Mahraoui et al. (1994) using Caco-2 cells. Treatment of the cells with forskolin, which stimulates adenylate cyclase and raises intracellular cAMP levels, increased fructose uptake 2-fold, and increased GLUT5 protein and mRNA 5-fold and 7-fold, respectively. Matosin-Matekalo et al. (1999) used Caco2 cells transfected with a GLUT5 promoter inserted up-stream of the luciferase reporter gene. They found that a region of the GLUT5 promoter binds the thyroid horomone receptor/retinoid X receptor heterodimers, and that both triiodothyroninr (T3) and glucose increase GLUT5 mRNA.

Helliwell et al. (2000a) looked at the regulation of GLUT5 by a number of signals that have well-established roles in the regulation of sugar transport. Isolated loops of rat jejunum were perfused with activators and inhibitors of the ERK, p38 and PI3K pathways. The findings suggest that the p38 pathway stimulates fructose transport, while the ERK and the PI3K pathways had little effect on fructose transport. Extensive cross-talk occurs between the pathways. For example, inhibiting the ERK pathway with PD98059 increased the sensitivity to anisomysin, which stimulates the p38 pathway. The authors concluded that the three pathways have the potential to regulate fructose transport during the digestion and absorption of a meal. They suggested that future work should focus on determining the hormones that influence these pathways, and the molecular mechanisms that regulate the levels and activities of the sugar transporters.

Gouyon et al. (2003a) used Caco-2 cells to investigate the mechanism by which fructose increases GLUT5 expression. Although both glucose and fructose increased the activity of the GLUT5 promoter, the effect of fructose was stronger and associated with higher cAMP concentrations. If cAMP signalling was blocked by a protein kinase A inhibitor, extensive GLUT5 mRNA degradation occurred, suggesting that the mRNA stability was influenced by PKA. A sugar response element was identified in the GLUT5 promoter. PABP-interacting protein 2, which represses translation (Khaleghpour et al., 2001a; Khaleghpour et al., 2001b), was identified as a component of GLUT5 3'-UTR RNA-protein complex, where it may act to destabilize transcripts. The differences between the effects of glucose and fructose on GLUT5 expression may be attributed to variations in their ability to increase cAMP levels, and to modulate the formation of protein complexes with GLUT5 3'-UTR.

Infection may also regulate fructose transport. Intravenous administration of Tumor necrosis factor-α (TNF-α) in rabbits significantly reduced jejunal fructose transport and GLUT5 protein (Garcia-Herrera, 2004). This inhibition was related to the secretagogue effect of TNF-α, and both nitric oxide and prostaglandins were implicated in the inhibition of fructose uptake. Adaptive immunity also influences the expression of a number of developmentally regulated genes. In mice lacking in adaptive immunity (B cell deficient recombination-activating gene [RAG] mice), RNase protection assays demonstrated that GLUT5 was increased (Jenkins et al., 2003).

Recent advances have been made in understanding the signalling pathways involved in the regulation of GLUT5. Cui et al. (2004) have demonstrated that cAMP stimulates fructose transport in the neonatal rat intestine. Perfusing fructose (100mM) plus 8-bromo-cAMP in 22 day old rats increased fructose uptake rates, while an inhibitor of adenylate cyclase abolished this effect. Despite the presence of two cAMP response elements in the human GLUT5 promoter region (Mahraoui et al., 1994), GLUT5 mRNA was not affected by cAMP treatment. Interestingly, inhibitors of PKA did not prevent the fructose-associated increases in transport, suggesting that cAMP modulates fructose transport independent of PKA (Table 3).

Subsequent work by the same group has shown that fructose-induced increases in neonatal rat intestinal fructose uptake involve the PI3K/Akt signalling pathway (Cui et al., 2005). In this study, PI3K inhibitors (wortmannin and LY94002) and an Akt inhibitor (SH-5) abolished the increase in fructose uptake, as well as the abundance of GLUT5 protein (but not mRNA) seen following fructose (100mM) perfusions in neonatal rats. Fructose perfusion increased phosphatidylinositol-3,4,5-triphosphate (PIP3), the product of PI3K, in the mid to upper regions of the villus, where most of the GLUT5 was located. The authors suggest that the PI3K/Akt pathway may be involved in the synthesis and/or recruitment of GLUT5 to the BBM in response to luminal fructose (Figure 2).

GLUT2

GLUT2 is a low affinity, high capacity facilitative transporter in the BLM that transports glucose, fructose, galactose and mannose (Thorens et al., 1988; Thorens et al., 1990; Cheeseman, 1993; Maenz and Cheeseman, 1987; Cheeseman and Tsang, 1996). It has 12 transmembrane domains, with intracellular N and C terminals. Using immunohistochemistry, Thorens et al. (1990) showed that GLUT2 expression increases as enterocytes migrate up from the crypt to the villous tip. Amino acid sequences in transmembrane segments 9-12 are primarily responsible for GLUT2's distinctive glucose affinity, whereas amino acid sequences in transmembrane segments 7-8 enable GLUT2 to transport fructose (Wu et al., 1998).

Luminal sugars (Cheeseman and Harley, 1991; Miyamoto et al., 1993) or vascular infusions of glucose or fructose (Tsang and Cheeseman, 1994; Shirazi-Beechey, et al., 1994) stimulate GLUT2 expression and activity. The response of GLUT2 to dietary sugars was investigated by Miyamoto et al. (1993). In this study, they fed sugar-enriched diets to male Sprague Dawley rats for 5 days. GLUT2 mRNA was up-regulated by glucose, fructose and galactose. GLUT2 modulation required intracellular metabolism of the sugar, as it was unaffected by 3-O-methyglucose, a non-metabolized glucose analog.

In a study by Cui et al. (2003), the jejunum of 20 day old anaesthetized rat pups was perfused with 100 mM glucose or fructose. Increases in GLUT2 mRNA were observed, and this effect was inhibited by actinomycin D, an inhibitor of transcription. Cycloheximide, an inhibitor of translation, did not block the enhanced expression of GLUT2 mRNA, suggesting that the synthesis of new proteins is not necessary for increases in GLUT2 mRNA. Because levels of GLUT2 mRNA and protein are tightly correlated, the regulation of GLUT2 may be transcriptional (Ferraris and Diamond, 1997).

PASSIVE UPTAKE

For years there has been considered to be a "passive" component to sugar absorption. This traditional view has been challenged, with the suggestion that the kinetic characteristics of sugar uptake could also be described by a second high affinity, high capacity BBM transporter (Thomson et al., 1987c). In order to better understand the new "GLUT2 trafficking model", we need first to consider the classic "passive permeation" model.

The fact that SGLT1 saturates at 30-50 mM glucose was inconsistent with the observation that intestinal glucose absorption increases linearly with increases in luminal glucose concentrations up to several hundred millimolar (Fordtran and Inglefinger, 1968). This finding suggests the presence of two components: an active, phloridzin-sensitive component, and a phloridzin-insensitive, possibly passive component that does not appear to be saturable. Some studies have suggested that the "passive" component played a large role in glucose transport at high glucose concentrations, in some models contributing 3-5 times as much as the active component (Ilundain et al., 1979; Lostao et al., 1991).

The passive component of glucose transport was characterized by Pappenheimer and Reiss (1987). The observation that high rates of water absorption accompany glucose absorption (Lifson and Parsons, 1957) provided a rationale for proposing that glucose in the intercellular spaces provided an osmotic force that resulted in bulk flow of nutrients. Pappenheimer and Reiss (1987) perfused isolated segments of hamster small intestine with 10-25 mM glucose. Structural studies using electron microscopy and freeze fracture analysis revealed large dilatations within junctions following glucose perfusion. They concluded that Na^+-coupled transport of solutes from the intestinal lumen to the cytosol of the enterocytes provides the driving force for the absorption of fluid and nutrients, and triggers the widening of intercellular junctions, thereby promoting the bulk absorption of nutrients by solvent drag. They calculated that the contribution of solvent drag exceeds that of active transport at luminal glucose concentrations greater than 250mM. Madara and Pappenheimer (1987) further demonstrated that the transport of glucose via SGLT1 caused dilatation of the tight junctions. They concluded that passive glucose absorption is a result of paracellular solvent drag, and is indeed SGLT1 dependent. This theory suggests a non-specific route, which could potentially allow passage of several solutes.

Ferraris and Diamond proposed an alternative theory, in which paracellular flow is negligible (Ferraris and Diamond, 1989; Ferraris et al., 1990). Based on the determination of up-dated kinetic constants for glucose absorption, and the determination of the usual free glucose concentrations in the intestinal lumen, they concluded that SGLT1 fully accounts for glucose absorption. Much of their work is based on studies examining long-term dietary adaptations, from which they concluded that BBM transporters are matched to dietary intake. Their model is supported by the findings of Lane et al. (1999), who demonstrated that paracellular flow in unanaesthetized dogs did not account for more than 2-7% of total absorption.

Much of the controversy surrounding the role of the paracellular pathway stems from the discrepancies between the estimated concentrations of glucose in the intestinal lumen. Pappenheimer and Reiss (1987) based their calculations on luminal glucose concentrations of ~300 mM, whereas Ferraris et al. (1990) did a detailed analysis of luminal glucose concentrations and concluded that physiological luminal values ranged from 0.2-48 mM. Pappenheimer (1993) used the rate of membrane hydrolysis of maltose to indirectly measure luminal glucose concentrations. They also point out that the techniques used by Ferraris et al. (1990), which involve glucose analysis of luminal contents, will underestimate the concentration found at the membrane following hydrolysis by disaccharidases. The actual physiological levels of glucose in the lumen remain a subject of debate.

The concept of more than one transport system for glucose was suggested by Malo (1988). Using human fetal and adult BBM vesicles, curvilinear Eadie-Hofstee plots and sodium activation curves were obtained when glucose concentrations were varied in the medium. These findings, coupled with determinations of phloretin-sensitive and -insensitive components, and the ability of the BBM vesicles to transport 3-O-methylglucose, suggested the presence of two transport systems: a high-affinity low-capacity system and a low-affinity high-capacity system (Malo, 1988; Malo, 1990). This agrees with the observation that Na^+/glucose cotransport saturates at 30-50 mM, yet absorption is linear from 50 mM to several hundred mM (Fordtran et al., 1968).

Although this concept was proposed many years ago, it was not until recently that interest in the area has re-emerged due to an alternative model of intestinal glucose transport proposed by George Kellett and his colleagues at the University of York, and by Edith Brot-Laroche and her colleagues at the University of Paris. Let us briefly explore this fascinating “voyage of discovery”.

GLUT2 IN THE BBM

Several years ago, GLUT2 was detected in the BBM of enterocytes in diabetic animals, although at the time this was interpreted to be a pathological event (Corpe et al., 1996). More recently, Kellett and his colleagues proposed a model by which BBM SGLT1, in the presence of luminal glucose, promotes the rapid insertion of GLUT2 into the BBM via PKCβII and the MAP kinase-dependent signal transduction pathways (Helliwell et al. 2000a,b; Kellett and Helliwell, 2000). PKCβII is located in the terminal web of mature enterocytes in the upper part of the intestinal villus (Saxon et al., 1994). Interestingly, these are the same cells that are responsible for glucose absorption.

Using a luminal perfusion model, Kellett and coworkers measured the phloretin-insensitive (SGLT1) component and phloretin sensitive component (GLUT2) of glucose transport. They also showed using Western blotting that BBM GLUT2 increased 2.2 fold when the concentration of glucose in the perfusate increased from 0 to 100 mM. Similarly, the BBM level of PKCβII increased with increasing glucose concentrations. This finding, coupled with the observation that PKCβII shows a saturation response and has a Km similar to that of SGLT1 (21-27 mM), suggests that PKCβII is an important signal in the recruitment of GLUT2 to the BBM. PKCβII levels also correlate with levels of GLUT2 in the BBM, offering further support for its role in the recruitment of GLUT2 to the BBM. The ability of the PKC inhibitor "chelerythrine" to block phorbol 12-myristate 13-acetate (PMA)-stimulated fructose transport and GLUT2 abundance in the BBM also supports this model (Helliwell et al., 2000b).

Kellett's working hypothesis proposes that before a meal, when luminal concentrations of glucose are low, GLUT2 levels in the BBM are also low, which would minimize the escape of glucose from the cell (any glucose that did escape would be recycled by SGLT1, which can transport it against the glucose gradient). Once a meal is ingested and BBM enzymes hydrolyse disaccharides, luminal glucose concentrations increase. Glucose uptake via SGLT1 causes increases in enterocyte volume due to a rise in osmolarity (and the co-transport of water molecules by SGLT1), and may trigger the entry of Ca+, activating PKCβII and promoting the insertion of GLUT2 in the BBM. The involvement of SGLT1 in the recruitment of GLUT2 to the BBM agrees with observations that phloridzin (an SGLT1 inhibitor) fully blocks glucose uptake, and that patients with defective SGLT1 suffer from glucose-galactose malabsorption. Trafficking of GLUT2 is thought to be rapid, with a $t_{1/2}$ of less than 5 minutes. A rounding of the apical surface, due to a contraction of the peri-junctional actomyosin ring, allows luminal glucose to have increased access to the BBM enzymes and transporters. The authors recognize that there may also be an activation of the small amount of GLUT2 that is already present in the BBM.

Helliwell et al. (2000a) investigated the role of several signalling pathways in intestinal fructose absorption. Using an *in vivo* perfusion model, they showed that fructose transport was mediated by both GLUT5 and GLUT2. Using PMA to stimulate a 4-fold stimulation of fructose transport, they saw a 4-fold increase in GLUT2 protein in the BBM that correlated with PKCβII activation. Only minor changes in GLUT5 levels were observed, suggesting that recruitment of GLUT2 to the BBM represents a mechanism by which absorptive capacity is matched to dietary intake.

Helliwell et al. (2003a) also established a role for the PI-3K and the mTOR pathways in the phosphorylation, turnover and degradation of PKCβII. Using an *in vivo* perfusion model, they showed that inhibitors of these pathways (wortmannin and rapamycin, respectively) block GLUT2 trafficking to the BBM and inhibit sugar absorption. A role for insulin in the regulation of intestinal sugar absorption is suggested. In their model, they suggest that as sugar absorption increases, the plasma sugar concentration increases, stimulating the release of insulin, which activates PI 3-kinase, resulting in the phosphorylation of PKCβII.

They also proposed a model by which amino acids promote the formation of competent PKCβII by activating the mTOR pathway, which prevents dephosphorylation of PKCβII (Figure 3). Thus, the dynamic control of intestinal sugar absorption may be achieved by the rapid turnover and degradation of PKCβII.

Why haven't previous investigators been able to detect GLUT2 in the BBM? *In vivo* endogenous hormones and nutrients activate PKCβII. Kellett's group points out that the process of harvesting tissue for *in vitro* preparations causes the inactivation of PKCβII and the rapid trafficking of GLUT2 away from the BBM. This observation may help to explain why the passive component was more apparent in the *in vivo* studies, as compared to *in vitro* experiments. In order to minimize the loss of GLUT2 from the BBM, Kellett's group perform all stages of tissue harvesting and membrane vesicle preparations at 0-4°C after perfusing the intestine with a sugar load.

Helliwell and Kellett (2002) looked at perfusion conditions in order to determine if the so called passive component was SGLT1-dependent, as suggested by their work, or was SGLT1-independent, as suggested by earlier work by Debnam and Levin (1975). They concluded that the passive component is independent of the active component in high mechanical stress perfusions, suggesting that SGLT1-dependent recruitment of GLUT2 did not occur under these conditions. This may be related to the restrictions in blood flow and supply of endogenous nutrients and hormones caused by the high stress perfusions.

However, under conditions of low mechanical stress, inhibition of SGLT1 with phloridzin does decrease GLUT2 levels in the BBM. Clearly, the perfusion conditions affect the results of the experiment, and this may explain the discrepancies between various studies. Finally, Kellett and colleagues suggest that the term "facilitated" should be used rather than "passive" to more accurately describe the GLUT2 mediated component of sugar uptake.

The antibody used to detect GLUT2 is also critically important in being able to detect GLUT2 in the BBM. Currently, there are two commercially available antibodies that recognize GLUT2: one that recognizes the extracellular loop between transmembrane 1 and 2 (Biogenesis, Poole, England); and another that recognizes a portion of the C- terminus (Research Diagnostics, Flanders, NJ). The choice of antibody is important, as Au et al. (2002) demonstrated that the biotinylation procedure they used to detect surface proteins interfered with the ability of the GLUT2 antibody to recognize the extracellular loop, forcing them to use the C-terminus antibody. However, Thorens et al. (1990) were unable to detect GLUT2 in the BBM by immunohistochemistry using an antibody that recognizes the C-terminus. In contrast, when using the antibody directed against the extracellular loop, Cheeseman's group was able to detect BBM GLUT2 (Au et al., 2002). In contrast, when using Western blotting on BBM vesicles, they were able to detect GLUT2 using either of the antibodies. Clearly, the choice of antibody depends on what method of detection is used.

Although earlier studies established a role for GLP-2 in modulating GLUT2 activity in the BLM (Cheeseman and Tsang, 1996; Cheeseman and O'Neill, 1998), more recently Au et al. (2002) investigated the effect of GLP-2 on the transient expression of GLUT2 in the BBM. Using an *in vivo* perfusion model in rats, they showed that a one hour vascular infusion of GLP-2 (800 pM) doubled the rate of fructose absorption, and this enhanced absorption of fructose could be blocked by phloretin, an inhibitor of GLUT2. Immunohistochemistry localized GLUT2 to both the BBM and BLM, and identified a pool of transporter located just under the microvilli in the terminal web region. This raises the possibility that the cytoskeleton structure is involved in the insertion of GLUT2 into the BBM. Luminal glucose perfusion (50 mM) or vascular GLP-2 infusion (800 pM) increased GLUT2 in the BBM two-fold as determined by Western blotting of biotinylated surface proteins. Both Kellett and Cheeseman suggest that in addition to the insertion of GLUT2 in the BBM, the intrinsic activity of apical GLUT2 may also be regulated (Kellett, 2001 and Au et al., 2002). The concept of intrinsic activity and its regulation will be discussed in subsequent sections.

Gouyon et al. (2003b) used confocal microscopy and immunofluorescence in mice to confirm the presence of GLUT2 in the BBM following five days of feeding a 65% sugar meal, or following an oral bolus of either fructose or glucose. Wild type and GLUT2 null mice were fed fructose, glucose or sucrose (65% glucose, fructose or sucrose)-rich diets for five days, or were fasted and then received a 40% fructose, glucose or sucrose bolus. The absence of GLUT2 did not significantly affect fructose absorption in animals fed a low-carbohydrate diet, suggesting that under these conditions GLUT5 is soley responsible for fructose uptake into the enterocyte.

In animals fed a high fructose diet, cytochalasin B (an inhibitor of GLUT2) inhibited fructose uptake 60% in wild type mice, whereas GLUT2 null mice were unaffected. A 40% reduction was observed in animals fed a high glucose diet. This suggests that under these conditions fructose enters the cell by both a cytochalasin B dependent process (GLUT2) and a cytochalasin B independent process (GLUT5). The trafficking of GLUT2 to the BBM may represent a mechanism by which sugar absorptive capacity is matched to dietary intake.

When GLUT2 null mice were challenged with oral fructose, transport was 60% lower than in wild type animals, indicating firstly that the absence of GLUT2 limited fructose uptake, and secondly that this could not be fully compensated for by GLUT5. Still, some compensatory changes were noted, as GLUT5 mRNA was found to be increased three-fold in the ileum, possibly indicating some fructose malabsorption in the GLUT2 null mice. Therefore, Gouyon's study demonstrates that while under control conditions GLUT5 is solely responsible for BBM fructose uptake, BBM GLUT2 may be responsible for ~40-60% of sugar uptake when the luminal sugar concentrations are high.

The role of AMP-activated kinase (AMPK), an intracellular energy sensor, in the regulation of intestinal sugar uptake has also been examined. Walker et al. (2004) demonstrated that the activation of AMPK resulted in the recruitment of GLUT2 to the BBM and a down-regulation of the energy-requiring SGLT1-mediated glucose uptake. The importance of this phenomenon, particularly in models of intestinal damage or stress, warrants further investigation.

The presence of GLUT2 in the BBM of humans has not been confirmed. Dyer et al. (2002) found GLUT2 expression was restricted to the BLM in humans, although critics of this work have speculated that this may be a result of the methods used to obtain and process biopsies, and the lack of feeding a high sugar diet or giving an oral sugar test prior to obtaining the biopsies (Gouyon et al., 2003).

In summary, the role of GLUT2 in the transport of sugars across the BBM remains controversial. The most recent data suggests that in specific situations, such as when luminal sugar concentrations are high, GLUT2 is recruited to the BBM and contributes to sugar uptake from the lumen. This may explain the discrepancies between studies and the inability of many researchers to detect GLUT2 in the BBM in their experimental models.

INTRINSIC ACTIVITY AND TRANSPORTER TRAFFICKING

A number of factors are involved in the regulation of intestinal sugar transport. These factors may modify sugar transport by altering the abundance of sugar transporters in the intestine. Alternatively, sugar transport may be regulated at an entirely different level. The intrinsic activity of the transporters (amount of substrate transported per unit of transporter protein) may be altered, in the absence of detectable changes in transporter abundance. Indeed, there has been a long history of reports of discrepancies between glucose uptake and the protein abundance of glucose transporters both in skeletal muscle (reviewed in Furtado et al., 2002), adipose (Barros et al., 1997) and in the intestine (Maenz and Cheeseman, 1986; Corpe et al., 1996; Helliwell et al., 2000a; Au et al., 2002 Thiesen et al., 2003b; Thiesen et al., 2003d; Drozdowski et al., 2003a; Drozdowski et al., 2003b). Changes in the intrinsic activity of glucose transporters have observed with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000) and following the activation of MAPK and PI3K (Helliwell et al., 2000a). The post-translational mechanism by which intrinsic activity is regulated is not known, but may involve phosphorylation or dephosphorylation of the transporter or the activation or inhibition of the transporter by a regulatory protein.

Kellett and his colleagues have shown that the PI3K pathway is involved in the modification of the intrinsic activity of GLUT2 and GLUT5 (Helliwell et al., 2000a). Control of transport by the modulation of both the levels and activities of the transporters occurred as a result of extensive cross-talk between the extracellular signal-regulated kinase (ERK), p38, and phosphatidylinositol 3-kinase (PI 3-kinase) pathways. Activation of the p38 pathway stimulates fructose transport by increasing GLUT2 levels in the BBM, as well as increasing the intrinsic activity of GLUT2. In contrast, the ERK or PI 3-kinase pathways have regulatory effects on transporter trafficking and intrinsic activity, without having significant effects on fructose transport (Figure 4). However, these results are derived from independently modulating these pathways, when clearly there is extensive cross-talk. For example, when the ERK pathway is inhibited, fructose transport stimulated by the activation of the p38 pathway increases 50-fold, suggesting that the ERK pathway restrains the p38 pathway.

It is not known if PI3K/Akt modifies the intrinsic activity of SGLT1. However, a study by Alexander and Carey (2001) showed that orogastric IGF-1 treatment increased glucose uptake in piglets without increasing SGLT1 abundance, suggesting an effect on intrinsic activity of the transporter. Inhibiting Akt blocked the increase in glucose uptake, possibly by modifying the activity of the transporter.

PI3K has also been implicated in the regulation of GLUT4 trafficking to the plasma membrane in adipocytes or muscle (reviewed in Furtado et al., 2002). Despite this possibility, several studies have demonstrated that the trafficking of transporter protein to the BBM cannot fully explain changes in intestinal sugar uptake seen after IGF-1, GLP-2 or glucose administration (Alexander and Carey et al., 2001; Au et al., 2002; Khoursandi et al., 2004). Nevertheless, both alterations in trafficking and intrinsic activity may contribute to the changes seen in sugar uptake. Further work is required to further characterize the relative contributions of each of these mechanisms.

ALTERNATIVE THEORIES

The previously well-accepted role of GLUT2 as the sole BLM glucose transporter is also a subject of debate. The role of GLUT2 was originally based on it being immunolocalized to the BLM. However, this does not exclude the possibility of other basolateral transport pathways. Recently, GLUT2 null mice were developed, in which GLUT1 or GLUT2 was re-expressed in pancreatic β cells to enable survival. This was an important step in investigating the role of GLUT2 in sugar transport. In these animals, normal rates of glucose appearance in the tail vein blood were seen following an oral glucose load, suggesting that GLUT2 was not required for transepithelial glucose transport (Thorens et al., 2000). It is important to note that this paper has limitations, as the appearance of glucose in the tail vein is not a direct measure of intestinal sugar transport. Further work by Stumpel et al. (2001) using an isolated intestinal perfusion model demonstrated normal glucose transport kinetics despite a lack of GLUT2. This finding was noted under control conditions and following cAMP perfusion, which is known to increase glucose absorption via SGLT1 (Stumpel et al., 1998). Even with this accelerated apical uptake of glucose into the enterocyte, the basolateral transport of glucose did not appear to be rate-limiting.

Interestingly, sugar transport was dose-dependently inhibited by an agent that inhibits the glucose 6-phosphate translocase located in the endoplasmic reticulum (ER) membrane. Glucose 6-phosphate translocase transports glucose-6-phosphate from the cytosol into the lumen of the ER, where the active site of glucose-6-

phosphatase is located. Furthermore, 3-O-methylglucose, which cannot be phosphorylated by the hexokinases, was not transported, despite the fact that it is a known substrate for both GLUT2 and SGLT1. Taken together, these findings suggest that a distinct pathway exists that involves glucose phosphorylation, transport to the ER, dephosphorylation, and release via a membrane-traffic based pathway (Figure 5). Interestingly, the expression of the glucose-6-phosphatase and the glucose-6-phosphate translocase, as determined by Northern blotting, were not increased in the GLUT2 null animals. This contrasts with the work of Gouyon et al. (2003b), who used RT-PCR to demonstrate that GLUT2 null mice had increased mRNA expression of glucose-6-phosphatase.

Stumpel and colleagues (2001) also noted that GLUT5 mRNA expression was increased in the GLUT2 null mice, while the expression of all other known GLUT transporters did not change. Human studies have demonstrated the presence of GLUT5 in the BLM of enterocytes (Blakemore et al., 1995). The finding that fructose absorption was unaffected by GLUT2 status suggests that GLUT5 may have been present in the BLM, contributing to fructose release on the serosal surface of the enterocyte. However, the authors dismissed the possibility that fructose and glucose shared a common serosal transport system based on the observation that the release of glucose, but not fructose, was blocked by an inhibitor of the glucose 6-phosphate translocase.

Stumpel et al. (2001) also performed fructose perfusion experiments in GLUT2 null mice. The results showed that intracellular fructose was not converted to glucose, further supporting the notion that this alternative pathway does not contribute to fructose efflux. The authors also discounted the possibility that the paracellular pathway significantly contributed to glucose absorption, as the SGLT1 inhibitor phloridzin greatly reduced glucose absorption. They concluded that a microsomal membrane traffic-based mechanism may be an important component of transepithelial glucose transport.

The investigators point out that the concept of a microsomal membrane-trafficking transport system is supported by the following observation: genes for glucose-6-phosphate translocase (G6PT1) (Ihara et al., 2000) and glucose-6-

phosphatase (G6PC) (Rajas et al., 1999) are expressed in human intestinal cells, despite the fact that only minimal amounts of glycogen are found in jejunal biopsies (Milla et al., 1978). Similarly, the high levels of hexokinase activity in intestinal cells (Newsholme and Carrie, 1994) support the concept of an alternative transport system characterized by glucose phosphorylation and subsequent microsomal transport and trafficking.

Santer et al. (2003) re-evaluated the role of GLUT2 in intestinal sugar absorption in one patient with Fanconi Bickel syndrome (FBS). FBS is characterized by congenital GLUT2 deficiency. Oral glucose tolerance tests performed on this patient failed to demonstrate differences in breath hydrogen concentrations when compared to control subjects, indictating that sugar was not being malabsorbed, at least within the sensitivity limits of hydrogen breath testing. These findings also suggest that other mechanisms are in place to transport sugars across the basolateral membrane of enterocytes.

RECENT DISCOVERIES

The model of intestinal sugar transport is an ever-changing story. Recently, a new facilitative glucose transporter, GLUT7, has been cloned and characterized (Li et al., 2004). GLUT7 has a high affinity for glucose (Km=0.3 mM) and fructose (IC50=0.060 mM), but not for galactose. GLUT7 mRNA is present in the human small intestine, colon, testis and prostate. GLUT7 protein was found in the intestine, mostly in the BBM. The transporter's high affinity led the researchers to speculate that it may be important in fructose absorption at the end of the meal, when concentrations of fructose in the intestinal lumen are low. The physiological relevance of GLUT7 is unknown, as it doesn't appear to compensate for the loss of SGLT1 in glucose-galactose malabsorption.

Tazawa et al. (2005) have also cloned SGLT4, a sodium-dependent glucose transporter found in the intestine, liver, and kidney. SGLT4 is a low-affinity protein that transports mannose. Furthermore, glucose, fructose and galactose were able to inhibit the transport of α-methyl-D-glucopyranoside in COS7 cells expressing SGLT4, suggesting that these sugars may also be substrates. Because mannose is elevated in diabetes (Pitkanen, 1996) and in the metabolic syndrome (Pitkanen et al., 1999), the authors suggest that SGLT4 may be a potential therapeutic target for patients afflicted with these disorders. Further characterization of these novel intestinal transporters will add to understanding of intestinal sugar transport.

The process of intestinal sugar absorption remains a controversial topic. An increased understanding of this process will enable the development of better therapeutic strategies in conditions where the modulation of intestinal sugar transport could improve health. For example, reducing sugar absorption may be beneficial with regards to the treatment of diabetes or obesity. Conversely, stimulating sugar absorption may be desirable in patients with short bowel syndrome, or in malnourished elderly patients. Furthermore, the targeted delivery of drugs to tumour cells expressing glucose transporters is an exciting area of research that warrants further exploration.

2.2 INTESTINAL ADAPTATION

The intestine has an inherent ability to adapt morphologically and functionally in response to internal and external environmental stimuli. In fact, intestinal adaptation may be considered as a paradigm of gene-environment interactions. The array of phenotypic adaptations includes the modification of brush border membrane (BBM) fluidity and permeability, as well as up- or down-regulation of carrier-mediated transport. Intestinal adaptation occurs following the loss of a major portion of the small intestine ("short bowel syndrome", SBS), following chronic ingestion of ethanol, following sublethal doses of abdominal irradiation, in diabetes, with aging, and with fasting and malnutrition (Thomson and Wild, 1997a; Thomson and Wild, 1997b; Ferraris and Carey, 2000). Following intestinal resection, morphological and functional changes occur depending upon the extent of the intestine removed, the site studied, and the lipid content of the diet (reviewed in Thiesen et al., 2003a). The increase in nutrient absorption compensates for the loss of absorptive surface area, and minimizes the malabsorption that could otherwise potentially occur. Therefore, intestinal adaptation has important implications in the survival potential and welfare of the host (Sturm et al., 1997). However, in some cases such as diabetes, intestinal adaptation may have deleterious effects, with enhanced nutrient uptake exacerbating prevailing hyperglycemia, hyperlipidemia and obesity (Burant et al., 1994)

The mechanisms of intestinal adaptation occur at a variety of levels: physiological, cellular and molecular. Signals of adaptation may relate to various hormone levels, transcription factors, ATP levels, or changes in the concentration of luminal solutes (Ferraris and Carey, 2000). The signals and mechanisms of the adaptive process may be different for the jejunum and ileum, as well as in the intestinal crypt and villous tip, explaining the site-specific alterations and differences between crypt and villous enterocytes (Thomson and Wild, 1997a; Thomson and Wild, 1997b).

Rodents are commonly used in well-characterized models of assessing the process of intestinal adaptation (Wolvekamp et al., 1996). Following small bowel resection in the rat, the remnant intestinal mucosa undergoes compensatory alterations in an attempt to restore normal absorptive capacity (O'Connor et al., 1999). Morphologic and functional changes include increases in crypt depth and villous length, enterocyte proliferation, as well as increased electrolyte, glucose and amino acid uptake (O'Connor et al., 1999; Wolvekamp et al., 1996).

The adaptive process has been defined in terms of transport kinetics. Changes usually occur in the value of the maximal transport rate (Vmax) rather than in the Michaelis affinity (Km) constant of specific nutrient transporters (sugars and amino acids) (Diamond et al., 1984; Ferraris and Diamond, 1989). Furthermore, there may be alterations in the passive permeability coefficients of nutrients transported passively such as short-, medium- and long chain fatty acids and cholesterol (Thomson and Wild, 1997a; Thomson and Wild, 1997b; Ferraris and Diamond, 1997). The increased Vmax results from either an up-regulation of the total number of transporters, an increased number of transporting mucosal cells, or an increase in the intrinsic activity fo the transporter (Kellett and Helliwell, 2000; Helliwell et al., 2000a). Intestinal resection also selectively changes the passive permeability properties of the BBM, as demonstrated by the increased uptake of fatty acids following intestinal resection, an increase that was not due to the changes in the mucosal surface area or the effective resistance of the intestinal unstirred water layer

(UWL) (Thomson et al., 1986). Indeed, this altered permeability is due to changes in the lipophilic properties of the BBM due to variations in the lipid content of the BBM (Keelan et al., 1985).

Intestinal adaptation in the rodent model of chronic diabetes involves changes at the transcriptional as well as the posttranscriptional level, leading to increased Na^+-coupled sugar absorption (Wild et al., 1999). After inducing acute hyperglycemia in rats, there is rapid up-regulation of glucose transport across the basolateral membrane (BLM) of the enterocyte (Cheeseman and Maenz, 1989). In this model, both the vascular as well as luminal glucose infusion causes an increase in the glucose transport capacity across the BLM (Tsang and Cheeseman, 1994). However, no significant increase in BLM cytochalasin B binding or in GLUT2 protein abundance was observed, suggesting that there may be a post-translational event that increases the number of GLUT2 proteins available for transport, such as the movement of GLUT2 to the BLM from a preformed pooln within the enterocyte. Alternatively, the "intrinsic activity" of the transporter may be altered in the absence of changes in the protein abundance. Changes in the intrinsic activity of glucose transporters have been observed with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000) and following the activation of MAPK and PI3K (Helliwell et al., 2000a).

Following extensive intestinal resection, there is hyperplasia of the remaining bowel, which may be accompanied by the enhanced uptake of nutrients (Dowling and Booth, 1967). The alterations in the cell kinetics that result in modification of the nutrition status may be specific or non-specific. *Non-specific mechanisms* involve alterations that result in changes in the intestinal mucosal mass and/or the villous surface area, leading to modifications in the uptake of all nutrients, including those that are absorbed passively (Rand et al., 1993). On the other hand, *specific mechanisms* involve up- or down-regulation of transporters responsible for the uptake of particular nutrients, such as sugars or amino acids (Thomson and Wild, 1997a; Thomson and Wild, 1997b).

The observation that morphological modifications may accompany intestinal adaptation in the rodent small bowel resection model was first made by Dowling and

Booth (1967). The remaining intestine after resection is hyperplastic, with greater villous height and crypt depth, leading to enhanced, mucosal surface area. However, while enhanced nutrient absorption is observed, the morphological changes do not necessarily explain the alterations in nutrient uptake. For example, one week after an 80% small bowel resection, the remaining intestine increased its mass to 50-70% of its pre-resection level, yet the uptake of glucose increased only to approximately 33% of the pre-resection level (O'Connor et al., 1999). Thus, enhanced nutrient absorption may not be solely explained by intestinal hyperplasia and/ or hypertrophy.

It is clear that dynamic morphologic parameters of the intestine may also adapt. For instance, the crypt cell production rates or the enterocyte migration rates change in some situations of intestinal adaptation (Thomson et al., 1994). It is important that morphological alterations be considered when estimating the kinetic parameters of absorption. Morphological modifications such as blunting of the mucosal growth or mucosal hyperplasia after intestinal resection are observed when Dexamethasone (Dex) is given subcutaneously (Park et al., 1994). Both kinetics and dynamic morphologic parameters are altered in the adaptive process, and the influence of resection on nutrient uptake is due in part to these kinetic alterations. This may be due to the altered cell kinetics changing the population of the enterocytes along the villus, thereby leading to variations in the number of cells with transporter, or the activity of the transporters (Smith, 1981; Fedorak et al., 1991).

Many models of intestinal adaptation have been described: glucose uptake has been found to be increased during pregnancy (Musacchia and Hartner, 1970), lactation (Cripps and Williams, 1975), with the ingestion of a high carbohydrate diet (Sanderson and Naik, 2000), hyperglycemia (Fischer and Lauterbach, 1984), with diabetes (Keelan et al., 1985), high alcohol intake (Dinda and Beck, 1981) and after intestinal resection (Robinson et al., 1982). On the other hand, glucose uptake is decreased with aging (Drozdowski et al., 2003), external abdominal radiation (Thomson et al., 1983), and with the use of total parenteral nutrition (Diamond and Karasov, 1987). Most transporters are up-regulated by the levels of dietary substrate levels, and yet toxic substances and essential amino acids have the opposite effect (Karasov and Diamond, 1983; Karasov et al., 1987; Diamond and Karasov, 1987;

Wolvekamp et al., 1996). These examples illustrate the diversity and variability of this intestinal adaptive process.

The adaptive response in humans is not well characterized. Increases in nutrient absorption have been documented (Pullan, 1959; Weinstein et al., 1969; Howard and Hassan, 1998) in humans following resection. The role of morphological changes in this process, however, has not been conclusively demonstrated. Remnant small bowel lengthening and dilatation has been noted in patients with SBS, suggesting morphologic mechanisms in human intestinal adaptation (Thompson et al., 1995). However, the mucosal adaptation typical in rodent models is not seen in the human adaptive response (Ziegler et al., 2002; Pironi et al., 1994). Indeed, several studies have shown that no increases in villous height or crypt depth were detected among patients who underwent intestinal resection, as compared to healthy controls (Porus, 1965; Weinstein et al., 1969).

With the existence of various relevant anatomical, physiological and biochemical differences between the human and rodent gastrointestinal tracts (Kararli, 1995), and a conspicuous lack of comparable human studies, the clinical adequacy of the rat as a model of intestinal adaptation remains to be determined. Although the morphological and functional changes that occur in the rodent following massive small bowel resection have been well characterized (Dowling and Booth, 1967; Gleeson et al., 1972), direct evidence for similar changes in humans is lacking. Accordingly, caution must be used when attempting to extrapolate findings from rodent studies to the human population. An alternative model, the neonatal piglet, has been used in short bowel studies (Benhamou et al., 1997; Heemskerk et al., 1999; Lauronen et al., 1998). The neonatal pig has recently been used to determine the effects of IGF-1 and dietary manipulations in an intestinal resection model (Heemskerk et al., 2002; Bines et al., 2002). The degree to which the results obtained using this model reflect human findings has yet to be determined, and the rodent remains a popular model for studies of intestinal adaptation.

DIETARY REGULATION

The topic of the dietary regulation of intestinal gene expression has been reviewed (Sanderson, 1998; Sanderson and Naik, 2000). Dietary constituents provide continual environmental signals that elicit the expression of a host of genes that influence intestinal adaptation (Jump and Clarke, 1999). Every day, enterocytes are exposed to different nutrients that vary according to the nutrient intake of the host. For this reason, the intestine must be able to adapt to variations in the dietary load and composition (Diamond, 1991; Sanderson and Naik, 2000). The intestine, like many other biological and engineered systems, is quantitatively matched to prevailing peak loads with modest reserve capacities. Indeed, physiological capacities are optimal and most economical if they ascribe to the adage "enough, but not too much" (Diamond, 1991). Therefore, intestinal enzymes and transporters are characterized by a "safety factor", a parameter that represents the ratio of its capacity to the load placed on it (Lam et al., 2002). The maintenance of this reserve capacity is biosynthetically costly, but is necessary given the unpredictable nature of dietary contents.

Parenteral vs. Enteral Nutrition

Small bowel atrophy is well characterized in rodent models using total parenteral nutrition (Levine et al., 1974; Johnson et al., 1975; Hosoda et al., 1989). Not surprisingly, the presence of luminal nutrients also contributes greatly to the adaptive process. Intestinal adaptation following massive small bowel resection is limited, but not entirely abolished in the absence of luminal nutrition (Koruda et al., 1988). The following sections detail the effects of the type and amount of various luminal nutrients on the adaptive process.

Lipids

Dietary fat content influences the uptake of hexoses and lipids into rabbit jejunum following ileal resection (Thomson et al., 1986). More recently, using a rat model of SBS, Sukhotnik et al. (2003) demonstrated that early feeding of a high fat diet increased lipid absorptive capacity of the intestinal remnant. The main mechanisms of this effect may be an acceleration of structural intestinal adaptation, resulting in an increased number of enterocytes. However, at the molecular and cellular level, a high fat diet decreased mucosal mRNA levels of the lipid binding protein FAT/CD36 and decreased oleic acid uptake by isolated enterocytes. This is in contrast to what is seen with the liver fatty acid binding protein (L-FABP), a cytosolic lipid binding protein. Mice that were chronically fed a diet enriched in sunflower oil had increased the liver fatty acid binding protein (L-FABP) mRNA levels in their small intestine (Niot et al., 1997). The effect was specific to this gene, as the intestinal fatty acid binding protein (I-FABP) was unaffected.

Not only the amount of fat, but also the type of dietary fat may influence intestinal function. Keelan et al. (1996) tested the hypothesis that the intestinal morphology and uptake of nutrients after resection of the distal half of the small intestine of rats responds to alterations in the dietary content of saturated (SFA) and polyunsaturated (PUFA) fatty acids. Adult female Sprague-Dawley rats were subjected to a sham operation or to the surgical resection of the distal half of the small intestine. The animals were fed chow for 3 weeks, then either chow or isocaloric semisynthetic SFA or PUFA diets for a further 2 weeks. The *in vitro* jejunal uptake of glucose was twice as high in animals that had undergone resection and were fed SFA than in those fed PUFA. It was suggested that SFA was necessary in the diet to ensure that adequate adaptation takes place.

Thiesen and colleagues examined the effect of dietary lipids on lipid uptake in rats post-resection. Intestinal resection had no effect on the mRNA expression of early response genes (ERGs), proglucagon, or the ileal lipid binding protein (ILBP), but was associated with reduced jejunal mRNA for ornithine decarboxylase (ODC) and for the liver fatty acid binding protein (L-FABP) (Thiesen et al., 2002a). These resection-associated changes in gene expression were not linked with alterations in the intestinal uptake of long chain fatty acids or cholesterol. In animals undergoing intestinal resection and fed SFA or given control vehicle, there was a reduction in jejunal proglucagon mRNA expression as compared to those animals fed chow or PUFA. ODC mRNA expression in the jejunum of resected animals was reduced. Thus, dietary lipids modify the uptake of lipids in resected animals, and ODC and proglucagon may be involved in this adaptive response (Thiesen et al., 2002b).

The way by which dietary lipids alter gene expression and consequently change membrane composition and/or nutrient transport may be through the activation of peroxisome proliferator-activated receptors (PPAR), hepatic nuclear factor-4 (HNF-4), nuclear factor κ B (NFκB), and sterol response element binding proteins 1c (SREBP1c) (Jump and Clarke, 1999). By binding to these transcriptional factors, dietary lipids affect the rate of transcription and consequently the protein synthesis of nutrient transporters (Sanderson, 1998; Poirier et al., 2001). It is also known that PPARs belong to the superfamily of receptors that include the glucocorticosteroid receptor (GR) (Huin et al., 2000). When the locally acting glucocorticosteroid (GC) budesonide was administered concomitantly with SFA diet, the jejunal uptake of glucose was increased but the ileal uptake of fructose was reduced (Thiesen et al., 2003d).

It has been suggested that dietary lipids participate in signal transduction involving the activation of second messengers, such as cAMP, Ca^{2+} and diacylglycerol, thereby changing the mRNA expression (Huwiler et al., 2000). Studies with glycosphingolipid have revealed the importance of these lipids and their metabolites in signaling pathways via the tyrosine kinase-linked receptors, a signal system mediated by protein kinase C (PKC), mitogen activated protein kinase (MAPK), other kinases, as well as mediated by the cytosolic Ca^{2+} concentration (Hakomori and Igarahi, 1995). More recently, additional new signals involved in the adaptive intestinal response 3 days after a 50% intestinal resection have been identified by cDNA microarray analysis, such as small proline-rich protein 2, involved in wound healing; glutathione reductase, a gene involved in intestinal apoptosis; NF-2 family members, also involved in apoptosis; etoposide-induced p53-mediated apoptosis; basic Kruppe-like factor, a transcription factor that activates the promoter for IGF-1; and prothymosin-α, involved in cell proliferation (Stern et al., 2001; Erwin et al., 2000). These observations of altered expression of signals are useful to generate hypotheses that can be tested in future studies to establish whether these signals represent a primary or a secondary event.

The glycospingolipid, phospholipid, cholesterol and fatty acid composition of plasma membranes may be modified in mammalian cells (Spector and Yorek, 1985).

For example, Keelan et al. (1990) demonstrated that alterations in dietary fatty acid saturation influence intestinal BBM phospholipid fatty acid composition in rats. The investigators proposed that the previously reported diet-associated changes in active and passive intestinal transport are due at least in part to these alterations in the fatty acid composition in BBM phospholipids. A diet enriched with SFA is associated with increases in the saturation of BBM phospholipid fatty acids, while a diet enriched with PUFA is associated with an increase in the unsaturation of BBM phospholipid fatty acids (Thomson and Wild, 1997a; Thomson and Wild, 1997b). The degree of fatty acid unsaturation or saturation, as well as the cholesterol and ganglioside/glycosphingolipid content, are factors that influence the fluidity of the BBM (Alberts et al., 1994; Bertoli et al., 1981). Changes in the fluidity of the BBM may alter the permeation of molecules and nutrients through this barrier, as well as the conformation of binding sites on transporter proteins such as SGLT1, GLUT5 (Meddings et al., 1990). For example, alterations in BBM fluidity influence the passive uptake of lipids, as well as the carrier-mediated D-glucose uptake (Brasitus et al., 1989; Meddings et al., 1990). While enhancement of fluidity increases the uptake of lipids, fluidization of BBM from enterocytes located on the villous tip decreases the uptake of D-glucose to levels seen in the BBM from enterocytes located on the crypts (Meddings and Thiessen, 1989). The explanation for the effect of BBM fluidity on glucose uptake is unknown, but represents a potentially important post-translational process.

The lipid composition of cell membranes alters the passive permeability properties and transporter activity across the membrane (Spector and Yorek, 1985). The altered membrane lipid composition may act in part by changing the viscosity or fluidity of the membrane, including the microenvironment surrounding the transporter. Meddings (1989) compared *in vivo* membrane lipid permeability within the same intestinal region, under conditions where membrane physical properties were radically altered by feeding rats an inhibitor of cholesterol synthesis. Marked reductions in membrane fluidity were observed due to the replacement of membrane cholesterol with its precursor 7-dehydrocholesterol. Associated with these alterations was a pronounced reduction in membrane lipid permeability. Therefore, BBM membrane lipid permeability, *in vivo*, appears to be correlated with the physical properties of the bilayer.

Recently, two types of specialized microdomains in the BBM have been identified: lipid rafts and caveolae. These regions are important in signal transduction as well as lipid and protein trafficking (Simons and Ikonen, 1997; Anderson, 1998; Brown and London, 1998). They are enriched in saturated fatty acids, cholesterol and gangliosides (Anderson, 1998; Brown and London, 1998; Galbiati et al., 2001). Feeding rats a diet enriched with gangliosides increases jejunal glucose uptake (Birecki et al., MSc thesis, 2002). Feeding a ganglioside-rich diet increases the ganglioside content and decreases the cholesterol content in the intestinal mucosa, plasma, retina and brain (Park et al., 2005a). Similar changes in the lipid composition of intestinal microdomains, or lipid rafts, occur following ganglioside feeding (Park et al., 2005b). Although SGLT1 has been localized to these microdomains in renal epithelial cells (Runembert et al., 2002), it is not known if sugar transporters reside in intestinal BBM microdomains. If this is the case, local changes in membrane fatty acids may affect the activity of transporter by altering the configuration of the protein, potentially exposing or masking the transporter binding sites and thereby modifying nutrient uptake. In addition, gangliosides may influence intestinal sugar transport via their effect on pro-inflammatory mediators, many of which are known to influence intestinal sugar transport (Ottlakan, 1998; Hardin et al., 2000; Garcia-Herrara et al., 2004). For example, in rats challenged with lipopolysaccharide, ganglioside feeding reduced the production of intestinal platelet activating factor, PGE2, LTB4, as well as reduced plasma levels of IL-1β and TNF-α (Park et al., PhD thesis, 2005).

Carbohydrates

Dietary carbohydrate may induce the intestinal adaptive response by increasing the abundance of hexose transporters to facilitate a higher rate of sugar absorption (Diamond et al., 1984). In a murine model, intestinal glucose uptake was directly correlated with the dietary carbohydrate load (Karasov and Diamond, 1983; Solberg and Diamond, 1987; Sanderson and Naik, 2000). The effect of dietary carbohydrate on nutrient transporter abundance has been reported in several animal models. For instance, the abundance of SGLT-1 in BBM and GLUT2 in the BLM were elevated in animals fed a high carbohydrate diet and associated with this enhanced level of protein was an increase in glucose absorption (Cheeseman and Maenz, 1989; Ferraris and Diamond, 1992; Shu et al., 1997). As well, the GLUT5 transporter abundance was elevated with enhanced dietary fructose, leading to increased fructose uptake (Brasitus et al., 1989).

The initiation of the dietary glucose-induced adaptive response occurs in the intestinal crypts, where the transport capacities of the nutrient transporters are programmed (Solberg and Diamond, 1987; Karasov and Diamond, 1983; Karasov et al., 1987; Shu et al., 1997). In this mouse model, phlorizin binding was utilized as a means of measuring the glucose transporter site density. Changing the murine diet from a high to a low carbohydrate regimen reduced the amount of glucose transporter, as estimated from the density of phlorizin binding. The alteration in the density of phlorizin binding was first observed in the crypt cells, and over a three-day period was subsequently seen in the villous tip cells. This suggests that the crypt enterocytes respond to the high carbohydrate diet to increase their phloridzin binding; those cells then migrate up the villous over the next three days, contributing to the process of enhancing glucose uptake.

The enterocytes may adapt to the high carbohydrate diet by increasing the crypt cell turnover rate, enhancing the enterocyte migration rate, as well as by reprogramming the capability of nutrient transporters in the crypts to accommodate to the requirement for higher monosaccharide transporters (Shu et al., 1997).

Animals fed a glucose-enriched diet have an increased glucose uptake, resulting from up-regulation of both BBM and BLM glucose transporters (Cheeseman and Maenz, 1989; Cheeseman and Harley, 1991; Ferraris and Diamond, 1992). The precocious introduction of dietary fructose causes enhanced expression of fructose transporters and fructose transport earlier during development, without changing glucose uptake (Shu et al., 1997). The substrates glucose and fructose are both specific in terms of up-regulation of their corresponding transporters, SGLT1 and GLUT5. Therefore, increasing the sugar composition of the diet results in increases in the transport of these nutrients. In contrast, increases in essential amino acids or other substances which are potentially toxic at high levels (such as iron, calcium or phosphorous) are associated with no change, or even reductions in transport (Thomson and Valberg, 1971; Diamond and Karasov, 1987).

Furthermore, in many cases other nutrients may be equal, or even more potent, inducers of the transporter than its specific substrate. For example, young animals fed a diet enriched with polyunsaturated fatty acids (PUFA) have a decline in glucose uptake, as compared to animals fed a saturated fatty acid (SFA) enriched diet (Thomson et al., 1988; Thomson et al., 1991; Thiesen et al., 2003d). Similarly, Vine et al. (2002) studied the effect of varying fatty acids on the passive and active transport properties of rat jejunum, and found that an SFA-enriched diet increased Na^+-dependent glucose uptake when compared to a diet enriched with n6 PUFA. In contrast, in aged rats, glucose uptake is increased by PUFA and not by SFA (Drozdowski et al., 2003).

Dietary fiber also modulates intestinal nutrient uptake. For example, a diet enriched with fermentable fiber increased glucose uptake and GLUT2 transporter abundance in dogs (Massimino et al., 1998). *In vitro* studies, in which rat intestinal tissue was incubated with β-glucan isolated from barley or oats, show reductions in the uptake of stearic and linoleic acids (Drozdowski et al., 2005, unpublished observations). Furthermore, many studies have investigated the effect of TPN supplemented with short chain fatty acids, the products of fiber fermentation. Increases in glucose uptake, GLUT2 mRNA and protein, and intestinal morphology were seen in normal rats as well as in rats following intestinal resection (Tappenden et al., 1997; Tappenden and McBurney, 1998; Tappenden et al., 1998; Bartholome et al., 2004).

Protein

Dietary protein also has an impact on the intestinal morphology and active amino acid transport (Scharrer, 1972; Karasov et al., 1987). Both *in vitro* (Lis et al., 1972) and *in vivo* (Scharrer, 1972) rat experiments have shown that a high protein diet increases amino acid uptake in the jejunum. An alteration in the amount of dietary protein induces reversible adaptation of the non-essential amino acid transport rate (Casirola et al., 1994). Feeding a high protein diet to mice induces a 77 – 81% increment in the uptake of non-essential amino acids (Karasov et al., 1987), yet only a

32 – 61% increase for essential amino acids. On the other hand, a protein-deficient regimen reduces uptake of non-essential amino acids, such as aspartate and proline, and maintains or increases uptake for essential amino acids and alanine. Thus, the nature of the adaptive response depends upon the type of amino acid and the needs of the animal.

Glutamine is a key metabolic fuel for enterocytes, mediating cellular nucleic acid synthesis and proliferation. Parenterally fed rats demonstrate decreased atrophy of the intestinal mucosa following glutamine supplementation (Ziegler et al., 1996). Glutamine administration also normalizes the reduced levels of intestinal adaptation in rats receiving total parenteral nutrition (TPN) following intestinal resection (Tamada et al., 1993). It is noteworthy that some studies of oral glutamine supplementation in the rat have failed to document more than temporary mucosal proliferation (Wiren et al., 1996). This indicates that mechanistic differences that are intrinsic to the method of glutamine administration may exist, and suggests that these may be significant in regulating the adaptive response.

Other amino acids may inhibit intestinal adaptation. Sukhotnik et al. (2005) examined the effects of the parenteral administration of the nitric oxide precursor arginine to rats following 75% small bowel resection. Arginine supplementation was associated with lower cell proliferation indexes and greater enterocyte apoptosis. This observation led the investigators to conclude that arginine inhibits structural intestinal adaptation.

Polyamines

Polyamines are found in all eukaryotic cells (Pegg and McCann, 1982), and they play an important role in growth and differentiation (Tabor et al., 1976). Polyamines are obtained either from the diet, or via synthesis from ornithine (Dall'Asta et al., 1983). Uda et al. (2002) demonstrated that luminal perfusions of polyamines rapidly (in less than 5 min) enhance intestinal glucose uptake in rats, and increase BBM SGLT1 protein.

Polyamine synthesis or uptake may be an important event that initiates the adaptive hyperplasia seen in the intestinal remnant after partial small bowel resection. Enteral diets supplemented with ornithine alpha-ketoglutarate (OKG), a precursor for arginine, glutamine and polyamines, enhances intestinal adaptation in models of intestinal resection. (Czernichow et al., 1997; Dumas et al., 1998). Indeed, studies by both Tappenden et al. (1996) and Thiesen et al. (2002a) suggest that ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis, may mediate the adaptive process in rats that is stimulated by the administration of either glucocorticosteroids or short chain fatty acids to rats following intestinal resection.

The role of polyamines in the adaptation of the intestine during development has also been studied. Wild et al. (2005, unpublished observations) showed that in postnatal rats, oral spermidine treatment resulted in the precocious expression of the intestinal sugar transporters (SGLT1, GLUT2 and GLUT5), as well as ODC. This led the investigators to conclude that oral polyamines induce precocious maturation of sugar transporters, which may be mediated by alterations in ODC gene expression.

HORMONAL REGULATION

Glucocorticosteroids

In a model of extensive intestinal resection (50% enterectomy), the remaining proximal and distal intestinal remnants were adequate to assess the morphology and function at these sites (Ziegler et al., 1996; Diamond et al., 1984). The glucocorticosteroid prednisone had no effect on the intestinal uptake of glucose or fructose in these resected animals (Thiesen et al., 2003d). In contrast, the locally acting steroid budesonide increased by over 120% the value of the jejunal Vmax for the uptake of glucose, and increased by over 150% the ileal uptake of fructose The protein abundance and mRNA expression of SGLT1, GLUT5, GLUT2 and Na+/K+ APTase α 1 and β 1 did not explain the enhancing effect of budesonide on glucose and fructose uptake. Budesonide, prednisone and dexamethasone reduced the jejunal expression of the early response gene c-jun. In resected animals, the abundance of the mRNA of ODC in the jejunum was reduced, and glucocorticosteroids (GC) reduced the jejunal expression of the mRNA of proglucagon. These data suggest that the enhancing influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC and proglucagon, or other as yet unknown signals.

In contrast, the uptake of D-fructose by GLUT5 was similarly increased with budesonide and with prednisone. The increases in the uptake of fructose were not due to variations in the weight of the intestinal mucosa, food intake, or in GLUT5 protein or mRNA expression. There were no steroid-associated changes in mRNA expression of c-myc, c-jun, c-fos, of proglucagon, or of selected cytokines. However, the abundance of ileal ODC mRNA was increased with prednisone. Giving post- weaning rats four weeks of budesonide or prednisone in doses equivalent to those used in clinical practice increases fructose but not glucose uptake. This enhanced uptake of fructose was likely regulated by post-translational processes (Thiesen et al., 2003d).

Growth hormone

Growth hormone (GH) has been suggested as possessing pro-adaptive properties (Thompson, 1997). In rats and piglets, GH administration results in an increase in small bowel length and function per unit length (Ulshen et al., 1993). Hypophysectomized rats undergo mucosal hypoplasia of the small bowel, as well as a reduced adaptive response following resection that is restored by GH (Taylor et al., 1979). In contrast, transgenic mice expressing elevated levels of GH experience hypertrophy of the small intestine (Ulshen et al., 1993). IGF-1 expression in the small bowel is regulated by GH and is believed to induce enterotrophic effects following resection (Lund, 1998; Tamada et al., 1993). In a rat model of SBS, acute IGF-1 treatment of TPN fed rats produced sustained jejunal hyperplasia, and facilitated weaning from parenteral to enteral nutrition (Gillingham et al., 2003). GH administration to normal rats has been reported to have positive effects on mucosal growth and intestinal adaptation following massive resection (Benhamou et al., 1994), although contradictory data exists (Park et al., 1996; Ljungmann et al., 2000). Human and rabbit studies have indicated that increased nutrient transport activity devoid of morphologic changes may be the method of GH-induced intestinal adaptation (Iannoli et al., 1997).

GH administration has been shown to inhibit the liberation of glutamine from muscle during catabolic states in humans (Biolo et al., 2000), suggesting a possible role for combined GH and glutamine provision in adaptive bowel enhancement. Trials investigating any such synergism in the rat have yielded conflicting results.

Some studies have failed to demonstrate an additive effect of GH and glutamine in the enhancement of post-resection intestinal adaptation (Gu et al., 2002), while others have documented a positive synergistic effect (Zhou et al., 2001).

The mechanisms of action by which GH and/or glutamine may enhance the human adaptive process cannot be clearly surmised from the existing rodent data. Many studies have inherent deficits in terms of nutrient controls that could have contributed to the conflicting outcomes which have been described. The trophic effects of enteral nutrition on the adaptation process are well known (Thomson and Wild, 1997a). Studies evaluating the contribution of non-specific, nutrient-derived augmentation of the adaptation process, as well as the mechanisms of any such nutrient factor interactions, may be useful in defining more accurate and therapeutically applicable results.

Animal studies have confirmed the enhancing effect of GH on nutrient absorption (Thompson, 1997; Ulshen et al., 1993). For example, GH has been shown to enhance the absorption of amino acids using *ex vivo* human BBM vesicles (Zhou et al., 2001). An intestinal mucosal GH receptor has been described in rats and humans (Lobie et al., 1990), and GH promotes cell differentiation and clonal expansion of these differentiated cells (Green et al., 1985).

Human studies have suggested that the efficacy of GH and/or glutamine therapy in the adaptive response of the small bowel may be based heavily upon the clinical status of the patient (for example, the presence of a portion of the colon in continuity with the remaining resected small intestine) (Byrne et al., 1998). Evaluation of the effect of such variables in the rat may facilitate further understanding of the pathology and physiology of the bowel adaptation process, as well as more clearly defining positive predictive indicators of the bowel's ability to be rehabilitated. Furthermore, existing human data has indicated that the administration of high concentrations of GH can actually increase patient morbidity and mortality (Szkudlarek et al., 2000), demonstrating a primary need for equivalent clinical research in the testing of these factors.

In home parenteral nutrition (HPN)-dependent patients with SBS, the use of high dose recombinant human GH (0.4 mg/kg/day) in controlled (Szkudlarek et al.,2000; Scolapio et al., 1997) and uncontrolled studies (Byrne et al., 1995) has led to variable results. These patients were given glutamine supplements by mouth or parenterally, and their diet was modified. In the randomized, placebo-controlled study of Scolapio et al. (Scolapio et al., 1997), the subjects ingested a standardized 1500 kcal/day diet, which is clearly different from the hyperphagic diet consumed by many SBS patients (Messing et al., 1991), and which may contribute to the physiological adaptation that occurs in the remaining intestine after extensive resection. It is unclear whether glutamine is beneficial for the adaptive response in humans, and in rat models of SBS, it is unclear whether glutamine supplementation is efficacious for the adaptive process (Gu et al., 2001; Vanderhoof and Langnas, 1997). Furthermore, both a hyperphagic diet and the absence of malnutrition are needed for humans to achieve optimal intestinal adaptation (Thissen et al., 1994; Thompson et al., 1995).

When HPN-dependent patients with SBS were provided a usual *ad libitum* hyperphagic diet, and given low doses of GH (0.05 mg/kg/day) for three weeks, there was significant improvement in the intestinal absorption of energy (15%±5%), nitrogen (14%±6%) and carbohydrate (10%±4%) (Seguy et al., 2003). The increased food absorption represented 37% ± 16% of total parenteral energy delivery. Body weight, lean body mass, D-xylose absorption, insulin-like growth factor 1, and insulin-like growth factor binding protein 3 increased, whereas uptake of GH binding protein decreased. During treatment with GH, improvement in net intestinal absorption compared with placebo was 427±87 kcal/day, representing 19% ± 8% of the total energy expenditure required to obtain energy balance equilibrium in patients with SBS (Messing et al., 1991).

A review of the literature in this area by Matarese et al. (2004) noted that there were differences in gastrointestinal (GI) anatomy, dietary compliance, nutritional status, presence of mucosal disease, and diagnosis both within and between the studies. They concluded that "*administering recombinant human growth hormone alone or together with glutamine with or without a modified diet may be of benefit when the appropriate patients are selected for treatment*".

Insulin-like growth factor 1

Insulin-like growth factor 1 (IGF-1) also proved to be efficient in increasing intestinal adaptation following resection in rats. IGF-1 treatment following 70% jejuno-ileal resection attenuated fat and amino acid malabsorption (Lemmey et al., 1994) and increased total gut weight by up to 21%. The IGF-1 receptor was increased in the jejunum and colon due to resection. Resection also increased circulating IGF-binding proteins (IGFBP). IGF-1 treatment had no effect on IGF-1 mRNA or IGF-1 receptor density, but increased IGFBP5 in the jejunum. This increase in IGFBP5 was correlated with jejunal growth after IGF-1 treatment (Gillingham et al., 2001).

More recently, a study was conducted to determine the effect of IGF-1 on enterocyte kinetics following intestinal resection (Dahly et al., 2003). IGF-I treatment in resected rats significantly increased jejunal mucosal mass by 20% and mucosal concentrations of protein and DNA by 36 and 33%, respectively, above the response to resection alone. These changes reflected an increase in enterocyte proliferation and an expansion of the proliferative compartment in the crypt. No further decrease in enterocyte apoptosis, or increase in enterocyte migration, was observed (Dahly et al., 2003).

IGF-I treatment may also facilitate weaning from parenteral to enteral nutrition. After a 60% jejunoileal resection plus cecectomy, rats treated with recombinant human IGF-I (3 mg/kg body weight/day) or control vehicle were maintained exclusively with TPN for 4 days and were then transitioned to oral feeding. TPN and IGF-I were stopped 7 days after resection, and rats were maintained with oral feeding for 10 more days. Acute IGF-I treatment induced sustained jejunal hyperplasia, as suggested from the presence of greater concentrations of both jejunal mucosal protein and DNA, and was associated with the maintenance of a greater body weight and serum IGF-I concentrations (Gillingham et al., 2003).

A study was done using male transgenic mice with targeted smooth muscle IGF-1 overexpression (Knott et al., 2004). These animals and non-transgenic littermates underwent 50% proximal small bowel resection. The results showed that growth factor over-expression led to a unique mucosal response characterized by a persistent increase in remnant intestinal length, and an increase in mucosal surface area. Therefore, IGF-1 signaling from within the muscle layer may be important in resection-induced intestinal adaptation.

Epidermal growth factor

Epidermal growth factor (EGF) up-regulates intestinal nutrient transport (Opleta-Madsen et al., 1991). This effect is mediated by PKC and PI3K (Millar et al., 2002), and involves the redistribution of SGLT1 from microsomal pools to the BBM (Chung et al., 2002). After massive intestinal resection, endogenous EGF is increased in the saliva and is decreased in the urine (Shin et al., 1999). EGF stimulates intestinal adaptation after intestinal resection: the BBM surface area and the total absorptive area increased until day 10, and EGF treatment induced a further increase in BBM surface area (Hardin et al., 1999). In a study by O'Brien and colleagues (2002), mice underwent a 50% small bowel resection or sham operation, and were then given orally an epidermal growth factor receptor (EGFR) inhibitor (ZD1839, 50 mg/kg/day) or control vehicle for 3 days. ZD1839 prevented EGFR activation, as well as the normal postresection increases in ileal wet weight, villus height, and crypt depth. Enterocyte proliferation was reduced two-fold in the resection group by ZD1839. These results more directly confirm the requirement of a functional EGFR as a mediator of the postresection adaptation response. Interestingly, previous work has demonstrated that the EGFR is predominantly located on the BLM of enterocytes (Wong et al., 1999), but after small bowel resection the EGFR shows redistribution from the BLM to the BBM, with no change in the total amount of EGFR (Avissar et al., 2000). It is not known how this redistribution occurs. This is an important point, since modification of this process may represent a useful means to accelerate the intestinal adaptive process.

In a study by Knott et al. (2003), laser capture microdissection (LCM) microscopy was used to elucidate the specific cellular compartment(s) responsible for postresection changes in EGFR expression. Mice underwent a 50% proximal resection or sham operation, and after three days frozen sections were taken from the remnant ileum. Individual cells from the villi, crypt, muscularis and mesenchymal compartments were isolated. EGFR mRNA expression for each cell compartment was quantified using real-time reverse transcription polymerase chain reaction (RT-PCR).

EGFR expression was increased two-fold in the crypt after resection, directly correlating with the zone of cell proliferation. This supports the hypothesis that EGFR signaling is crucial for the mitogenic stimulus for adaptation. The additional finding of increased EGFR expression in the muscular compartment is novel, and may imply a role for EGFR in the muscular hyperplasia seen after massive small bowel resection. As noted previously, it is of interest that the muscle layer also appears to play a role in the adaptive response to IGF-1 (Knott et al., 2004)

The treatment of resected rats with EGF has been studied. In a study by Sham et al. (2002), male juvenile rats underwent either transection or ileocecal resection leaving a 20-cm jejunal remnant. Resected animals were treated orally with placebo or recombinant human EGF. Resected EGF-treated animals lost significantly less weight than those in the transection group, absorbed significantly more 3-0 methylglucose, and had reduced intestinal permeability as determined by the lactulose/mannitol ratio. Work by Chung et al. (2004) using rabbits showed that intestinal resection altered SGLT1 mRNA and protein expression along the crypt-villous axis, with expression being highest in the mid-villous region. Oral EGF normalized SGLT1 expression, resulting in a gradient of increasing expression from the base of the villus to the villous tip.

More recently, Nakai and colleagues (2004) investigated the role of EGF in stimulating intestinal adaptation following small bowel transplantation. Treatment of rats with EGF (intraperitoneally for three days) following intestinal transplantation resulted in increased glucose absorption, SGLT1 abundance and the villous height and crypt depth in the graft. This has not yet been studied in humans.

Keratinocyte growth factor

In a study by Yang et al. (2003), adult C57BL/6J mice were randomized to a 55% mid-small bowel resection, resection with keratinocyte growth factor (KGF) administration (SBSKGF), or a sham-operated (control) group, and were killed at day 7. Ussing chamber studies showed that KGF increased the net transepithelial absorption of 3-O-methyl glucose as well as sodium-coupled alanine absorption, but had no effect on epithelial permeability barrier function. Epithelial cells were separated along the crypt-villous axis with LCM, and epithelial KGF receptor

(KGFR) mRNA abundance was studied using real time RT-PCR. KGF up-regulated KGFR mRNA abundance, predominately in the crypt and the lower portion of the villus.

Leptin

Leptin plays an important role in the regulation of body fat and satiety (reviewed in Jequier, 2002). Leptin reduces food intake (Campfield et al., 1995) and leptin-deficient mice develop obesity (Zhang et al., 1994). Leptin may be a potential growth factor for the normal rat small intestine. The effect of 14 days of parenteral leptin administration (2ug/kg/day) to rats following 80% small bowel resection was studied. Leptin was associated with a 44% increase in galactose absorption and a 14% increase in GLUT-5 abundance, but with no change in DNA content or in SGLT abundance. These findings suggest that leptin may potentially be clinically useful in patients with impaired intestinal function (Pearson et al., 2001).

Ghrelin

Ghrelin is a gastric hormone that is released in response to enteral nutrients. It has an opposite effect when compared to leptin, as it stimulates food intake (Wren et al., 2001). The role of ghrelin in intestinal adaptation is unknown.

Glucagon-like peptide 2

Animal studies have demonstrated a potential role for GLP-2 in the adaptive response following intestinal resection (Dahly et al., 2003). Several investigators have demonstrated increases in plasma GLP-2 levels following intestinal resection in rats (Topstad et al., 2001; Thulesen et al., 2001; Ljungmann et al., 2001). In a study by Dahly et al. (2003), rats were subjected to 70% midjejunoileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP-2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats. While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 days post-resection. These data support a significant role for endogenous GLP-2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats (Dahly et al., 2003b).

Recently, further correlations between post-resection GLP-2 levels, morphological indices, crypt cell proliferation rates and nutrient absorption have been made (Martin et al., 2005). In this study, an inverse correlation was found between post-prandial GLP-2 levels and fat or protein absorption as assessed by a 48 hours balance study. These results, along with data obtained from studies showing that GLP-2 immunoneutralization inhibits post-resection adaptation (Perez et al., 2005), further implicate GLP-2 as a post-resection mediator of adaptation.

GLP-2 administration in rats increases the adaptive response to massive intestinal resection (Scott et al., 1998). In this study, Sprague-Dawley rats were divided into two groups, with a 75% mid-jejunum-ileum resection and a sham operated group. Animals were treated with 0.1 µg/g GLP-2 analog (protease resistant human GLP-2) or placebo given subcutaneously twice daily for 21 days. The groups were compared measuring the total weight of the rats, and the mucosal mass per centimeter. Administration of this peptide or its analogs was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum. The absorption and urinary excretion of oral D-xylose is proportional to intestinal mucosal surface area and transit time. While resection reduced D-xylose excretion, GLP-2 restored D-xylose excretion to levels above control values within 21 days of administration. This indicates that GLP-2 has a positive effect on intestinal morphology and absorptive function following resection.

More recently, Martin et al. (2004) investigated the effects of GLP-2 in a TPN-supported model of experimental short bowel syndrome. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, or TPN + 10 µg/kg/h GLP-2. TPN plus GLP-2 treatment resulted in increased bowel and body weight, villus height, intestinal mucosal surface area, crypt cell proliferation. Intestinal permeability tests showed that GLP-2 reduced the lactulose-mannitol ratio indicating that GLP-2 lowered intestinal permeability when compared with the TPN alone. GLP-2 increased serum GLP-2 levels and intestinal SGLT-1 protein abundance compared with either TPN or enteral groups. This study demonstrates that GLP-2 is capable of stimulating intestinal adaptation in the absence of enteral feeding, Because a number of hormones and growth factors have been shown to influence intestinal function, Washizawa et al. (2004) compared the effects of GLP-2, GH and KGF on markers of gut adaptation following massive small bowel resection (MSBR). KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in the small bowel and the colon. They also observed that while both GH and KGF increased colonic mucosal growth, GLP-2 exerted superior trophic effects on jejunal growth. GLP-2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP-2, GH and KGF on gut adaptation following MSBR, the authors conclude that a combination of these agents may be most beneficial.

A pilot study to determine the efficacy of GLP-2 in patients with SBS has been completed. A non-placebo controlled study was conducted in 8 patients with SBS with an end-enterostomy type of anastomasis (6 had Crohn's disease and 4 were not receiving HPN) (Jeppesen et al., 2001). Treatment with GLP-2 (400 μg subcutaneously twice a day for 35 days) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in 5 and 6 patients, respectively.

A review by Jeppesen (2003) on the role of GLP-2 in the treatment of SBS concludes that *"Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research studies. The optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of parenteral nutrition in a larger fraction of short-bowel patients".*

Signals of Intestinal Adaptation

A number of studies have investigated the signals involved in intestinal adaptation using animal models of intestinal resection. Dodson et al. (1996) identified three subsets of genes that were up-regulated by constructing a cDNA library from the remnant ileum of resected rats. This library was screened, and subtractive hybridization was used to identify genes that were induced following resection. These included genes involved with regulating the absorption and metabolism of nutrients. For example, L-FABP, apolipoprotein A-IV, cellular retinal binding protein II and ileal lipid binding protein were identified as genes that were induced following 70% intestinal resection in rats (Dodson et al., 1996). Genes involved in cell cycle regulation were also identified. For example, phosphorylation and dephosphorylation are important regulators of the cell cycle, and PP1δ, a subunit of a serine/threonine phosphatase was indeed up-regulated. Grp78, a member of the heat shock protein family was also increased. Grp78 resides in the ER and acts as a chaperone during protein assembly and transport. It may also have a protective role, and prevent apoptosis as a way of promoting the proliferative response following intestinal resection (Sugawara et al., 1993; Potten et al., 1994).

Rubin et al. (1998) further characterized the molecular and cellular mechanisms following 70% resection in rats. An immediate early gene, PC4/TIS7, was markedly increased soon after resection, with levels returning back to normal by one week post-resection. Although the function of this protein is unknown, it may be related to cytodifferentiation as it is expressed only in the villus and not in the crypts.

Erwin et al. (2000) used cDNA microarrays to gain insight into the mechanism of intestinal adaptation. Mice underwent a 50% intestinal resection, and three days afterwards RNA was extracted from the remnant ileum. Multiple genes were induced, and they fell into these four categories: 1) Apoptosis, DNA synthesis, repair and recombination (10 genes); 2) Oncogenes, tumor suppressors, cell cycle regulators (3 genes); 3) Stress response, ion channels and transport (4 genes); 4) Transcription factors and general DNA-binding proteins (1 gene).

Many of the genes (ODC, c-neu, glucose-related protein, IGFBP-4) that were identified agreed with the results of other studies of intestinal resection. For example, ODC was increased in this study, and this agrees with previous findings that showed ODC to be involved in the adaptive process (Rountree et al., 1992; Tappenden et al., 1996, Thiesen et al., 2002a). Some new factors were also identified including glutathione reductase (involved in apoptosis), Basic Kruppel-like factor (transcriptional regulator that activates the IGF promoter), prothymosin-α (associated with increased cell proliferation), and eteposide induced p53 responsive mRNA (stress response protein involved in p53 mediated apoptosis).

Stern et al. (2001) performed a similar analysis of gene expression following 50% intestinal resection in rats. The gene with the largest increase was identified as *sprr2*, a novel gene not previously known to be involved in intestinal adaptation. EGF administration post-resection further increased *sprr2* expression, and enhanced the adaptive response. This protein plays a role in the terminal differentiation of stratified squamous epithelium. Its role in the intestinal epithelium is unclear and warrants further investigation.

Finally, a variety of other signals have been described as possibly playing a role in the process of intestinal adaptation. These include prostanoids (Unmack et al., 2001), uncoupling proteins (Izadpanah et al., 2001), peroxisome proliferation – activated receptor α (PPARα) (Murase et al., 2001), transforming growth factor-α (Balasubramaniam et al., 2000), SPARC (secreted protein, acidic and rich in cystine (Puolakkainen et al., 1999), Bcl-2 (Vachon et al., 2001), endothelin-1 (Nankervis et al., 2001), erythropoietin (Juul et al., 2001), the GATA family of zinc finger transcription factors (Jonas et al., 2000), hepatocyte growth factor (Kato et al., 1998), the early response genes (ERG) (Sacks et al., 1995), PC4/TIS7 (Rubin et al., 1998) and epimorphin (Goyal et al., 1998). More recently, augmented Wnt signaling has been shown to enhance the adaptive response to massive small bowel resection (Bernal et al., 2005). Several of these signals may be useful to modify in a clinical setting to enhance the intestinal adaptive response.

Microarray technology is a powerful tool that is constantly developing into a more sophisticated technique of identifying novel genes involved in physiological processes. Intestinal adaptation awaits further characterization by hypothesis-testing studies. From the information that is available at this time, it is clear that genes regulating the cell cycle, proliferation, differentiation and apoptosis are important components of the adaptive process.

The process of intestinal adaptation is complex and multifaceted. Although a number of trophic nutrients and non-nutritive factors have been identified in animal studies, successful, reproducible clinical trials are lacking. Understanding the mechanisms underlying this adaptive process may direct research toward strategies that maximize intestinal function and impart a true clinical benefit to patients with short bowel syndrome.

2.3 ONTOGENY

At the time of birth, the human small intestine is morphologically and biochemically more mature than that of other mammals, including rats. The maturity of the small intestine is reflective of the length of the gestational period, with the development of the human small intestine being largely completed *in utero* by the end of the first trimester (Montgomery et al., 1999). Despite temporal differences in the ontogeny of the small intestine between species, the processes involved in the development of the small intestine remain similar. Thus, the human intestine goes through each of the stages that occur in rodents, so that animal studies may be used to better understand the development of the human intestine.

Development of the small intestine is comprised of three stages: 1) morphogenesis and cell proliferation; 2) cell differentiation; and 3) functional maturation (Colony, 1983). Gastrulation is the process by which the primitive gut tube is formed, consisting of the endoderm, the precursor to the epithelial lining of the GI tract, surrounded by mesenchyme. In humans this process begins at three weeks gestation (Montgomery et al., 1999).

The GI system is one of the first to polarize in the embryo by forming an entry and exit to the systems along the anterior and posterior axis. The *hox* genes are nuclear transcription factors that activate genes that encode secretory proteins. The *hox* genes play an important role in the formation of distinct regions of the brain and skeleton (Montgomery et al., 1999). Through epithelial-mesenchyme interactions, these proteins may also be involved in determining anterior-posterior patterning in the fetal gut. Similarly, Sonic hedgehog and Indian hedgehog pathways mediate epithelial-mesenchymal interactions at early stages of gut formation (reviewed in de Santa-Barbara et al., 2003).

A transition into columnar epithileum with the development of polarized enterocytes occurs, with the formation of apical, lateral and basal cell surfaces of the enterocyte. The formation of nascent villi occurs simultaneously, with cellular proliferation detectable along the villi. In humans, villous formation is initiated at 9-10 weeks gestation, and proceeds in a cranial-caudal direction (Montgomery et al., 1999). Villus and microvillus formation account for the 100,000 fold increase in the intestinal surface area observed from the early first trimester period to birth (Neu and Koldovsky, 1996).

The development of intestinal crypts then follows, with humans displaying early crypt development when compared to rodents, whose crypts do not develop until after birth (Hirano and Kataoka, 1986). Curiously, the human fetus and the neonatal rat have transient villus-like structures in the proximal colon with properties similar to enterocytes, including the expression of BBM enzymes and transporters (Foltzer-Jourdainne et al., 1989; Lacroix et al., 1984; Zweibaum et al., 1984).

In conjunction with the development of villi and crypts, epithelial lineages emerge giving rise to populations of enterocytes, goblet cells, enteroendocrine cells and Paneth cells. In addition to the four major cell lineages, M cells associated with Peyer's patches are detected by 17 weeks (Moxey and Trier, 1978). In the human intestine, all epithelial cell types known to occur in the adult are present by the end of the first trimester (Moxey and Trier, 1978). The intestinal epithelium is able to maintain differences in the differentiation programs of each lineage, depending on the location of the cells along the crypt-villous and proximal-distal gradients (Roth et al., 1991; Simon and Gordon, 1995).

The regulation of gastrointestinal development is complex, and involves a host of growth and transcription factors. Receptors for epidermal growth factor (EGF), transforming growth factor β (TGFβ), insulin-like growth factor II (IGF-II), hepatocyte growth factor (HGF), and GLP-2 are present in fetal human intestine (Podolsky, 1993; Lovshin et al. 2000). Human fetal cortisone levels in the blood increase late in gestation (Murphy, 1982), and corticosterone, a glucocorticoid similar to cortisol, is thought to be the main factor involved in rat small intestinal maturation (Marti and Fernandez-Otero, 1994; McDonald and Henning, 1992).

Studies investigating the development of human fetal small inestine xenografted to *scid* mice demonstrate that the transplanted intestine undergoes normal differentiation in the absence of luminal and hormonal factors (Winter et al., 1991; Savidge et al., 1995). This finding, in conjunction with the observation that villus formation in rodents is autonomous (Montgomery et al., 1981), suggests that intestinal development may be regulated largely by intrinsic factors ("hard-wired"), with extrinsic factors playing only a secondary role. Indeed, several transcription factors including *N-myc*, HNF3β and *Cdx-2* have been identified as potential intrinsic factors implicated in GI development. *N-myc* gene knockout animals demonstrate defects in GI development (Stanton et al., 1992). Homologous null mutants of HNF3β are lethal, as many structures, including the gut tube do not develop normally (Ang and Rossant, 1994; Weinstein et al., 1994). Cdx-2 expression is detected at the time of morphogenesis in mouse intestine, and is a known regulator of small intestinal sucrase-isomaltase (SI) expression (Suh et al., 1994). The exogenous expression of Cdx-2 in a rat intestinal cell line induces the differentiation of goblet and absorptive cells from crypt cells, suggesting a possible role in the ontogeny of the GI tract. Several other signalling pathways (including the Notch, Wnt/β-catenin and bone morphogenetic protein (BMP) pathways) are thought to play a role in patterning the gut during development and in regulating epithelial differentiation through epithelial-mesenchymal interactions (Roberts et al., 1995; Roberts et al., 1998).

In addition to regulation by transcriptional factors, intestinal development may be controlled through interactions with components of the extracellular matrix (ECM). Indeed, developmental changes in E-cadherins and integrins have been described (Basora et al., 1997; Rao et al., 1994; Simon and Gordon, 1995), suggesting that the ECM may influence the ontogeny of epithelial cells. Human fetal enterocyte cultures demonstrate enhanced differentiation when grown on ECM components (Sanderson et al., 1996), suggesting that a permissive rather than an instructive role may be attributed to the ECM in GI development. Indeed, mice in which many of the major components of the ECM have been deleted showed no changes in gastrointestinal morphogenesis, indicating that these components are not essential for GI development in this model (Hynes, 1996).

The functional development of intestinal brush-border membrane (BBM) enzyme activity in the small intestine has been well characterized (Seetharan et al., 1977; Vaeth and Henning, 1982; Martin and Henning, 1984; Leeper and Henning, 1990). In general, rodents are born at a more immature stage than are humans, and at least some of the BBM enzymatic maturation that occurs prenatally in humans only occurs after birth in rodents. This makes the rodent a useful model to use to better understand the process of intestinal maturation that occurs in premature infants. Significant differences are seen between species. For example, BBM SI is first detected in the human fetus in the first trimester, but is not seen until weaning in the rat (Leeper and Henning, 1990). Both rat and human fetal small intestine demonstrate detectable BBM lactase-phlorizin hydrolase (LPH) activity, however LPH expression before and after birth varies depending on the species studied (Montgomery et al., 1991).

In humans, BBM enzyme activity has been correlated to morphogenesis, with the development of enzyme activity being associated with the formation of enterocytes (Lacroix et al., 1984). Proximal-to-distal gradients of enzyme activity are established early. In addition, crypt-villous gradients are evident, with LPH activity being highest at the villous tip, and SI activity maximal in the mid-villous region (Goda et al., 1999).

LACTASE-PHLORIZIN HYDROLASE (LPH)

The major carbohydrate in milk is the disaccharide lactose. Lactose is cleaved by BBM LPH into glucose and galactose. LPH is therefore a crucial enzyme for neonates who are soley dependent on their mother's milk for nourishment.

Human LPH is first detected in the proximal small intestine at 8-9 weeks gestation, but later extends along the length of the small intestine (Lacroix et al., 1984). In contrast, rat LPH is very low until 24 weeks gestation, when activity begins to increase. A late gestational rise in LPH activity occurs in humans throughout the third trimester, while increased activity in rodents only occurs late in the third trimester (Figure 6). In the human fetal jejunum, LPH activity correlated with the abundance of mRNA (Villa et al., 1992), consistent with the belief that LPH activity is regulated transcriptionally (Escher at al., 1992; Wang et al., 1994; Fajardo et al., 1994). Nuclear transcription factors that have been shown to interact with the LPH promoter element CE-LPH1 include CDX-2 (Troelsen et al., 1997), HOXC11

(Mitchelmore et al., 1998), GATA6 (FitzGerald et al., 1998), and HNF1 (Mitchelmore et al., 1998).

Once weaning has occurred in rats, LPH activity declines, and this decline is associated with reduced LPH mRNA abundance (Colony, 1983; Motohashi et al., 1997). In fact, nearly all species of mammals lose LPH activity following weaning (Galand, 1989; Kodolvsky, 1981; Montgomery et al., 1991). Even in humans, the vast majority of the world's population experiences a decline in LPH activity sometime during childhood or adolescence to values 5-10% of those seen in early childhood (Buller and Grand, 1990). In contrast, in geographical regions such as Western Europe and North America, where for thousands of years dairy cattle were raised as a continuing source of milk, LPH activity usually persists throughout adulthood (Galand, 1989; Kodolvsky, 1981; Montgomery et al., 1991).

Human studies that investigated the correlations between mRNA abundance and activity of LPH suggest that transcriptional and post-transcriptional mechanisms are involved in the development of hypolactasia (Rossi et al., 1997). Post-translational mechanisms may also be involved in the decline in LPH activity through the modulation of functional protein along the villus. Glycosylation of the protein results in a 225 kDa form; however, the mature enzyme represents a cleavage product of this glycosylated precursor (Montgomery et al., 1991; Naim and Lentze, 1992; Danielsen et al., 1984). The initial cleavage occurs intracellularly in a post-Golgi compartment (Lottaz et al., 1992), and yields a product that lacks enzymatic activity. Once it is inserted into the BBM, LPH is once again cleaved, this time by extracellular trypsin to yield the mature and active 145 kDa form of the enzyme (Wuthrich et al., 1996).

SUCRASE-ISOMALTASE (SI)

SI is a bifunctional BBM disaccharidase with sucrase, isomaltase and maltase activity. Sucrase hydrolyzes sucrose into glucose and fructose. In humans, SI is first detect at 9-10 weeks gestation, and gradually increases until just prior to birth, when a marked increase occurs. After birth there is a rapid decline in SI levels to values comparable to those found in early gestation (Grand et al., 1976).

In mice, low levels of SI mRNA abundance are detectable in the small intestine (Tung et al., 1997). However, rat studies show that there is no BBM SI activity from birth until weaning (Leeper and Henning, 1990). At weaning, a dramatic increase in SI activity occurs, with adult levels being rapidly established. Expression of SI mRNA and protein is first detected in cells located at the crypt-villous junction, suggesting that the cells containing SI are programmed in the crypts. As these enterocytes migrate up the villus, the entire villus ultimately becomes populated with cells expressing SI. SI expression first appears in the proximal small intestine, and then proceeds distally to the ileum.

The dramatic increase of SI activity in rodents at weaning appears to be a genetically programmed event that is not significantly affected by the animals' diet (Henning, 1985; Kodolvsky, 1981). Premature SI induction, however, can be induced by stress, glucocorticosteroids, or by the thyroid hormone, thyroxine (Henning, 1985; Kodolvsky, 1981). Insulin may also regulate SI expression, as demonstrated by studies showing that low doses of insulin induce precocious SI activity and mRNA, an effect that may be reversed by treatment with antibodies to the insulin receptor (Buts et al., 1998).

Fetal human SI protein is in the proSI form from 15 to 30 weeks gestation, but after 30 weeks most of the protein consists of sucrase and isomaltase subunits (Triadou and Zweibaum, 1985). Enterokinase activity, which activates proteases that cleave proSI, appears at 26 weeks, and coincides with the appearance of the sucrase and isomaltase subunits.

SI is transiently expressed in the colon of both humans and rodents (Tung et al., 1997; Zweibaum et al., 1984; Lacroix et al., 1984; Foltzer-Jourdainne et al., 1989), in association with the appearance of small intestinal-like morphology. The observation that SI is expressed in colorectal cancer cells suggests that the factors that normally repress SI expression in the colon may be lost in cancer cells.

There is a correlation between fetal SI activity and mRNA abundance, suggesting that there is control at the level of transcription or mRNA stability (Sebastio et al., 1987). A number of regulatory elements (including SIF1, SIf2 and SIF3) have been identified within the promoter region of the SI gene, and are important for transcriptional induction. CDX-2 binds to the SIF1 element and transactivates the SI gene promoter (Suh et al., 1994). CDX-2 appears to be the major regulator of SI transcription, although a number of other potential transcription factors have been identified. For example, HOXC11, like CDX-2, binds to the SIF1 element of the SI promoter (Mitchelmore et al., 1998). HNF1α interacts with the SIF3 element and to a lesser extent SIF2, to activate SI transcription (Wu et al., 1994). GATA zinc-finger transcription factors interact with a region of the SI promoter upstream of the SIF1 element (Silberg et al., 1997).

Glycosylation of the SI protein occurs in the endoplasmic reticulum (ER) and in the Golgi apparatus, yielding a 245 kDa protein (Naim et al., 1988). Once the protein is inserted into the BBM, the cleavage of the molecule into two subunits occurs via trypsin digestion in the intestinal lumen (Naim et al., 1988), and the subunits remain associated by hydrostatic bonds. Defects in post-translational processing are thought to be responsible for inherited SI deficiency in humans, as alterations in glycosylation as well as intracellular transport have been observed (Fransen et al., 1991).

GLUCOSE TRANSPORT

The ontogeny of intestinal nutrient transport is largely dependent on the species that is studied. In all species, transporter protein does not appear until the intestine differentiates and forms crypts, villi and microvilli. The time at which this process occurs differs between species (please see above), and may be affected by the length of the gestational period. Differentiation of the mucosa alone, however, is not solely responsible for triggering the appearance of transporters, as many of them do not appear until after birth, or even after weaning.

Much of the research on the ontogeny of intestinal transport comes from rodent studies. When compared to humans, rodents are considered to be altricial, meaning that in many respects they are born premature. Indeed, many of the postnatal changes in the intestine seen in rats occur prenatally in humans, making neonatal rodents an ideal model for premature infants (Koldovsky, 1989). A number of researchers have also used the pig as a model of ontogeny due to the similarities between the pig and the human small intestine (Puchal and Buddington, 1992). In this review, we will present data largely from humans, rats and pigs in order to explain the overall pattern of intestinal ontogeny, irrespective of specific timelines.

The intestinal transport of nutrients, such as glucose, is first detected in the fetal small intestine of mammals, including humans (Rubin, 1992) (Figure 7). At this stage, both placental nutrients (Chabot et al., 1983; Chapman et al., 1974) as well as the swallowing of amniotic fluid (Mulvihill et al., 1985) contribute to fetal nutrient acquisition. In fact, the volumes swallowed in humans *in utero* at term are estimated to be approximately 750 ml/day (Pritchard, 1966).

Buchmiller et al. (1992) demonstrated that injection of galactose into the amniotic fluid of fetal rabbits increased intestinal mucosal weights as well as the uptake of both glucose and galactose in. This demonstrates that even fetal rabbits are able to up-regulate intestinal transport capacity in response to nutrients. Taste buds are detected early in gestation (Bradley and Mistretta, 1975), and early experiments have shown that human fetal swallowing increases following transamniotic saccharin infusion, and decreases following the infusion of a noxious substance (De Snoo, 1937; Liley, 1972).

The importance of fetal swallowing in the development of the GI tract is also highlighted by experiments in which fetal sheep underwent esophageal ligation to prevent amniotic fluid from reaching the small intestine (Trahair et al., 1986). A decrease in small intestinal villous height, intestinal weight and body weight resulted. Prenatal intestinal transporters are now considered to be critical for the development of the fetus, as an estimated 10-14% of fetal protein requirements in rhesus monkeys are met through nutrients that are present in the amniotic fluid (Pitkin and Reynolds, 1975). The presence of growth factors released from the gastrointestinal tract may also be important, as gastric infusions of epidermal growth factor (EGF) reversed the weight loss seen following esophageal ligation (Mulvihill et al., 1985).

Once the cells lining the intestine differentiate into columnar epithileum at 9-10 weeks of gestation, BBM proteins including SGLT1 are expressed (Buddington, 1994). Significant levels of SGLT1 mRNA are also detected in fetal tissue, suggesting that carrier-mediated transport of glucose may be occuring (Buddington and Malo 1996; Smith, 1981). Dramatic increases in the site density of SGLT1 are observed in fetal pigs between 74% of term and birth (Buddington and Malo, 1996). Between 17 and 30 weeks gestation, the duodenal-ileal gradient of glucose absorption is established in humans (Malo and Berteloot, 1991). In rats, glucose transport and SGLT1 protein and mRNA increase at weaning to levels higher than those seen in the suckling period or in adult animals (Khan et al., 2000; Shu et al., 1997). Curiously, phloridzin does not block glucose transport by SGLT1 in suckling and mature animals to the same extent that it does in weanlings. This may suggest the presence of an age-specific alternative mechanism of glucose transport, or an age-related difference in the phloridzin binding site on SGLT1.

In fact, detailed analysis of glucose uptake rates in BBM vesicles from human fetal tissue suggests the presence of two transport systems. In addition to SGLT1, a low affinity, high capacity system is detected in the proximal small intestine (Malo, 1988; Malo 1990). This may represent GLUT2, which has been described in the BBM of adult rats exposed to high luminal sugar concentrations (Kellett and Helliwell, 2000). However, an alternative pathway for intestinal glucose uptake may exist in the fetus. In adults, GLUT1 is expressed in erythrocytes and in the brain, with lower levels detected in adipose tissue, muscle and the liver (reviewed in Wood and Trayhurn, 2003). However, human and rat fetal small intestine also express GLUT1, which appears earlier than GLUT2, and decreases gradually during fetal life (Davidson et al., 1992; Matsumoto, et al.,1993). Although the mechanism of this developmental regulation is unknown, GLUT1 may be involved in early cell growth and proliferation.

Intestinal BLM GLUT2 mRNA is expressed at high levels at birth (Shu et al., 1997). In fact, GLUT2 mRNA is detected in fetal rats as early as day 16 following conception, even before intestinal villi are formed (Matsumoto et al., 1993). GLUT2 mRNA increases after weaning, and subsequently decreases to adult levels (Shu et al., 1997).

Cui et al. (2003) showed that GLUT2 in the developing intestine is regulated by luminal glucose and fructose. Luminal perfusions of 20 day old rat pups' intestines with fructose or glucose (100 mM) increased GLUT2 mRNA. This effect was blocked by the transcription inhibitor actinomycin D, but was not affected by the protein synthesis inhibitor cycloheximide. GLUT2 mRNA was also increased in bypassed intestinal loops, suggesting that systemic factors are involved in its regulation. Interestingly, GLUT2 mRNA abundance was even higher in the bypassed loop than in the section that was perfused, suggesting a possible compensatory mechanism due to perceived starvation.

Sugar uptake increases with the gestational age of the animal, and typically peaks immediately after birth, when the intestine takes over the burden of nutrient acquisition from the placenta. Studies done on pigs using the everted sleeve method demonstrate that the Vmax for D-glucose were highest immediately after birth before suckling, with a subsequent decrease associated with the onset of suckling (Puchal and Buddington, 1992). In newborn pigs, the onset of suckling appears to stimulate increases in BLM GLUT2 density (Cherbuy et al., 1997).

At birth, all enterocytes appear to have the capability to transport nutrients. As a result, uptake occurs in enterocytes from all along the villus, rather than just from the upper third, such as occurs in older rats (Smith, 1981). This may contribute to a higher rate of sugar uptake. Soon after birth, however, the lifelong gradient of increasing transport as one moves from the crypt to the villus is established, and may be partially responsible for the reduced uptake capacity of the intestine observed post-natally (Smith, 1981). The "dilution" of fetal enterocytes with new immature cells that do not express transporters may be responsible for this effect. Indeed, the subsequent age-related decline in transport observed in chickens was attributed to reductions in the site density of SGLT1 (Barfull et al., 2002).

Studies on human premature neonates have used the urinary excretion of D-xylose and 3-O-methyl-glucose as measures as passive and active carrier-mediated monosaccharide absorption, respectively. Neonates born between 28 and 32 weeks gestation showed increased absorption of these sugars when compared to those born before 28 weeks gestation (Rouwet et al., 2002).

Developmental changes in the intestinal transport of nutrients may also be non-specific (for example, changes in mucosal surface area, proliferation and migration of enterocytes, or changes in intestinal permeability). Indeed, the replacement of rat fetal enterocytes along the villus requires up to 2 weeks, as compared to the 24-48 hours required for the replacement of adult enterocytes (Smith and Jarvis, 1978). Non-specific changes are responsible for ontogenic alterations in mucosal weight, surface area and transport capacity. Postnatal development of enterocytes results in increases in the surface area of microvilli and the BLM (Morikawa et al., 1991; Vagnerova et al., 1997). Reduced turnover rates result in longer lifetimes of enterocytes, resulting in slower replacement of cells.

Paracellular permeability and BBM fluidity are age-dependent and may affect nutrient transport. Human neonates show decreases in intestinal permeability within the first 30 days of life as assessed by the lactulose/mannitol permeability test (Catassi et al., 1995). Reductions in fluidity occur in post-weaning rabbits, in association with increases in the cholesterol-to-phospholipid ratio in the microvillus membrane (Schwarz et al., 1989; Schwarz et al., 1984). In general, reductions in fluidity result in reductions in permeability.

FRUCTOSE TRANSPORT

Although SGLT1 and GLUT2 are expressed both in the fetus and at birth, the expression of BBM GLUT5 is delayed, and is only detected in post weaning rats (Castello et al., 1995; Rand et al., 1993; Shu et al., 1997; Toloza and Diamond, 1992). This contrasts with what is seen in pigs (Puchal and Buddington, 1992) and lambs (Char and Rudolph, 1979). In rats, GLUT5 protein and mRNA abundance parallel fructose transport, and therefore remain low throughout the suckling phase. GLUT5 protein and mRNA also remain low throughout weaning in rats, with higher levels detected in the post-weaning phase (Rand et al., 1993; Castello et al., 1995; Shu et al., 1997). This increase coincides with the appearance of fructose in the pups' diet, and parallels the increase in fructose uptake seen at this period. Although there is a temporal association between dietary fructose and the appearance of GLUT5, the expression of the transporter is "hard wired" and occurs at this time even in the absense of dietary stimuli (Shu et al., 1998). However, the precocious introduction of fructose to the diet of 22 day old rat pups can stimulate fructose transport and GLUT5 mRNA expression (Shu et al., 1998). Jiang and Ferraris (2001) showed that luminal perfusions of high concentrations (100 mM) of fructose resulted in increases in

GLUT5 mRNA and activity. This developmental reprogramming of fructose transport required *de novo* mRNA and protein synthesis, as both actinomycin D and cycloheximide (inhibitors of transcription and translation, respectively) abolished the effect.

The introduction of solid foods and fruit juice containing fructose at earlier stages of infancy, coupled with the increased use of fructose as a sweetener in dietary products, has resulted in increased exposure to fructose during infancy. Fructose has been implicated as the major cause of "toddler's diarrhea", largely because it is a late onset transporter that increases postnatally in human infants (Hoekstra et al., 1993). The infant intestine may not be equipped to absorb high amounts of fructose, resulting in fructose malabsorption. Fructose may then enter the colon, and the high osmolarity may cause in osmotic diarrhea. Even in adults, the incidence of fructose intolerance may be increasing: in a recent study published by Choi et al. (2003) patients with persistent, unexplained, non-specific GI symptoms received 50 grams of fructose. Samples were collected for breath hydrogen analysis. Positive results, indicating fructose malabsorption, were obtained in 73% of patients. Although the authors point out that the dose used in this study is considered to be high, it is approximately equivalent to the amount of fructose found in two cans of soda.

AMINO ACID TRANSPORT

Amino acid transporters appear prenatally in the intestines of chickens, rats, rabbits and humans, and these transporters increase dramatically in the first days after birth (Younoszai and Lynch, 1975). In rats, BBM amino acid transporters are expressed prenatally at the same time or shortly after SGLT1 (Buddington and Diamond, 1989). Using BBM vesicles, Malo (1991) characterized several amino acid transport pathways in 17-20 week gestation human fetal small intestine. All of the systems studied (neutral, acidic, basic and imino) were found to be functional, with a proximal-distal gradient established shortly after crypt-villus formation. Lebenthal and Lebenthal (1999) proposed a temporal sequence of GI tract development in humans that places amino acid transport at 14 weeks gestation, before glucose transport which occurs at 18 weeks gestation, and before fatty acid absorption which does not appear until 24 weeks gestation.

In rats, the highest rates of amino acid uptake are seen at birth, with decreases during suckling and postweaning (Navab and Winter, 1988). Age related changes in BLM amino acid transporters have not yet been described. Peptide uptake, like amino acid uptake, declines with age in rabbit (Guandalini and Rubino, 1982) and guinea pig intestine (Himukai et al., 1980).

Amino acid transporters, including NBAT (which transports cationic and neutral amino acids) and EAAC1 (which transports glutamate) are expressed in suckling rats (Rome et al., 2002). The transport of peptides occurs via PepT1, which is distributed throughout the small intestine. The distribution of these transporters in suckling rats parallels that seen in adult animals. NBAT mRNA is highest in the proximal small intestine, while EAAC1 mRNA was highest in the more distal regions. While a marked crypt-villous gradient was found for PEPT1 and NBAT, EAAC1 immunoreactivity is confined to the lower third of the villus and to the crypts, making it the first amino acid transporter with decreased expression during epithelial cell differentiation (Rome et al., 2002).

It is clear that developmental changes in amino acid and peptide transport occur. The ontogeny of amino acid transport is complicated due to major species differences, and by the large number of transport systems and individual amino acids, some of which are essential or non-essential depending on the age of the animal. Also, protein requirements change throughout the lifespan of an animal, necessitating variations in either intake or uptake of protein or amino acids. For example, intestinal proline uptake per mg of tissue was maximal at birth in rats, decreased at the end of the suckling phase, and decreased further in older animals. This decline matched both the dietary protein levels and protein requirements (Toloza and Diamond 1992). In addition, the decline in uptake of essential amino acids is greater than that for non-essential amino acids (Buddington and Diamond, 1989), as young animals have a disproportionate need for essential amino acids in early life due to the rapid growth rate at this age.

There are also different patterns of uptake for individual amino acids. For example, in cats, the basic amino acid transport declines more steeply than does neutral amino acid transport; in humans, lysine and phenylalanine transport appears later than the transport of alanine, leucine, taurine and valine (Moriyama, 1986). In rats, uptake declines at a similar rate with age for proline, methionine and lysine; however the decline in leucine uptake occurs twice as quickly (Buddington and Diamond, 1989). Finally, in rats a transition occurs at weaning when the uptake of glucose, fructose and lysine increase, coupled with decreases in proline and leucine uptake (Toloza and Diamond 1992).

MACROMOLECULE TRANSPORT

The uptake of macromolecules across the intestinal wall represents an important route by which immunoglobulins, growth factors and antigens are absorbed. This route of entry is especially important in neonates, who rely on it to obtain important immune factors from maternal colostrum or milk. Most species, including rats, mice and humans, are born hypoglobulinemic, and obtain IgG passively from maternal milk through proximal small intestinal absorption (Israel et al., 1997; Udall et al., 1984).

The transport of macromolecules across the BBM may occur by receptor-mediated or non-specific transcytosis. The effective transport of macromolecules is facilitated by the presence of protease inhibitors in the maternal colostrum (Westrom et al., 1985). Rodent studies demonstrate specific receptors that bind to the Fc portion of IgG (Jakoi et al., 1985). These receptors are transcriptionally regulated, and are present in the highest amount in the proximal duodenum (Martin et al., 1997). In humans, IgG is transferred from the placenta to the fetus in the third trimester of pregnancy, with receptors being detected in both the fetus and the neonate. This suggests that macromolecular transfer across the BBM persists after birth, and then gradually decreases (Israel et al., 1997; Axelsson et al., 1989). The high permeability of the intestine declines after birth, leading to a process commonly referred to as "gut closure". The time at which the macromolecular transport ceases varies between species, with a rapid decrease in transport observed in pigs within the first few postnatal days, and a similar decrease seen around 21 days after birth in rats and rabbits (Teichberg et al., 1990). In humans, the exact time that gut closure occurs is unknown, but intrinsic factors as well as growth factors, hormones and breast milk may play a role in regulating this process. The decline in permeability may also be related to changes in the mucus gel layer. Indeed, morphological analysis of intestinal tissue showed increased intervillus mucus gel in weaned as compared to suckling rats (Iboshi et al., 1996). This would potentially increase the effective resistance of the unstirred water layer, and thereby decrease the uptake of macromolecules.

Several studies have attempted to characterize macromolecule uptake in human infants. Jakobsson et al. (1986) used human alpha lactalbumin to demonstrate that macromolecule uptake declined with advancing postconceptual and postnatal age. Robertson et al. (1982) showed that preterm infants had higher serum beta lactoglobulin concentrations when compared to term infants who received this milk protein, providing indirect evidence to support the concept of "gut closure" in humans. The ability of the neonatal intestine to adapt to the presence or absence of luminal stimuli is apparent from studies demonstrating a delay in spontaneous closure of the intestine if breastfeeding is postponed beyond the first 30 hours of life (Vukavic, 1984). In this study serum IgA was used as an indicator of gut permeability to macromolecules.

LIPID AND BILE ACID TRANSPORT

The ability of the fetus and neonate to absorb lipids has been examined. Studies using human fetal jejunal explants (14-20 week gestation) maintained in serum free organ culture demonstrate increases in chylomicron, VLDL and HDL, paralleled by increases in triglycerides and cholesterol esters. This demonstrates the ability of the fetal intestine to absorb fat in conjunction with ontogenic increases in lipid and lipoprotein synthesis (Thibault et al., 1992). Apolipoprotein B synthesis is developmentally regulated. Fetal intestine synthesizes only apoB-100 at 11 weeks, but both apoB-48 and apoB-100 at 16 weeks, with apoB-48 being predominant in the mature intestine (Glickman and Magun, 1986).

The immature rat intestine is able to absorb fatty acids and cholesterol (Meddings and Theisen, 1989). However, pinocytosis of lipid globules is important after birth (Berendsen et al., 1979). Triglycerides are digested by gastric and lingual lipases into fatty acids and 2-monoacylglycerols, whose uptake is higher in the immature intestine when compared to adults (Flores et al., 1989; Meddings and Theisen, 1989). Although fatty acid binding proteins on the BBM are present in adults, lipid uptake is thought to be passive in sucklings (Meddings and Theisen, 1989). Lipids are assumed to be resynthesized into triglycerides (TG), phospholipids (PL) and cholesterol esters (CE), based on the presence of reesterification enzymes in the immature intestine (Shiau et al., 1979).

Bile acids are necessary for solubilizing lipids in the intestinal lumen. Intestinal bile acid uptake is an important step in the enterohepatic circulation of bile acids (recently reviewed in Kullak-Ublick et al. (2004), and this uptake is therefore important in the overall process of lipid absorption. Bile acid transporters are curiously absent during suckling when fat intake is high, and when bile acid secretion and recycling would be expected to be maximal. Passive absorption of bile acids during the suckling period may be the mechanism by which bile acids are recirculated (Stahl et al., 1993). Although the reasons for the absence of transporters during the suckling period are unclear, it is speculated that the malabsorption may allow bile acids to enter the colon and affect the development of the enteric flora (Sanderson and Walker, 2000).

Sodium-dependent bile acid transporters in the BBM or cytosol are detected in the rat at weaning (Christie et al., 1996; Little and Lester, 1980). Abrupt increases in bile acid transport at weaning occur in rat and human ileum, and are due to parallel increases in the steady state mRNA abundance and transporter number (Barnard et al., 1985; Barnard and Ghishan, 1987).

PANCREATIC ENZYMES

Despite their apparent ability to absorb lipids efficiently, preterm infants have higher fecal fat losses than do term infants. This is thought to be attributable to lower pancreatic and intestinal lipase activities. Despite the presence of lipase in breast milk, micellar absorption of lipids appears later when pancreatic lipase and bile acid concentrations increase (reviewed in Thomson and Keelan, 1986).

The human exocrine pancreas is functionally immature at birth, with substantial development occurring after birth. Proteolytic enzymes are detected early in the human fetus (20-25 weeks gestation), with each enzyme developing in a unique manner. Trypsin increases during fetal life, to reach 90% of childhood levels at term (Lebenthal and Lee, 1980). In contrast, at birth chymotrypsin and carboxypeptidase B levels are less than 60 and 25% of childhood levels, respectively. Despite the differences in the temporal development of proteolytic enzymes, protein digestion in the preterm neonate is adequate, and may be supported by the early development of gastric pepsin and mucosal peptidases (Keene and Hewer, 1929; Lindberg, 1966).

Amylase activity is negligible in the human fetus, and is not detectable until 1 month after birth. (Lebenthal and Lee, 1980). Salivary amlyase is detected at 20 weeks gestation, and while levels are low at birth, they increase to adult levels by the third month following birth (Sevenhuysen et al., 1984). Amlyase is also present in human milk, and may aid in the digestion of starch contained in weaning foods (Dewit et al., 1990). The reduced levels of amylase activity may reflect the low levels of starch in the neonatal diet. At weaning, an increase in amylase activity is detected (Lebenthal and Lee, 1980). While this may be influenced by the appearance of starch in the infant's diet, animal studies demonstrate a persistent increase in amylase activity in rats subjected to prolonged nursing (Lee et al., 1982). This suggests the presence of an inherent genetic program for the expression of amylase activity, not necessarily related to the starch content of the diet.

Pancreatic lipase activity in humans is detectable at 32 weeks gestation, remains low at birth, and increases 10 weeks after birth (Cleghorn et al., 1988). Lingual and gastric lipases, however, are detected at 26 weeks gestation (Hamosh et al., 1981). At birth these lipases are able to hydrolyse 60-70% of ingested fat, even in the absence of pancreatic lipase (Sheldon, 1964). Lipase and esterase activity is present in human milk, and contributes to the increased fat absorption observed in breast-fed infants (Alemi et al., 1981). While some authors have suggested that low lipolytic activity may be the rate-limiting step in the development of efficient fat absorption (Lebenthal and Lee, 1980; Hernell et al., 1989; Boehm et al., 1995), other authors propose that it is not the ability to hydrolyse triglycerides but rather the ability to take up long chain fatty acids (LCFA) from the lumen that is the rate-limiting step (Rings et al., 2002).

The ontogeny of the intestine is "hard wired", and occurs even in the absence of luminal and hormonal factors (Winter et al., 1991; Savidge et al., 1995). Still, a number of studies have demonstrated that variations in maternal diets, as well as weaning diets, can influence the ontogeny of the intestine (Thomson et al., 1989; Perin et al., 1997; Jarocka-Cyrta et al., 1998). "Critical period programming" is a phenomenon by which a biological mechanism is irreversibly turned on or off once during a lifetime in response to prevailing conditions at a critical stage (Karasov et al., 1985). This concept, which has also been referred to as "metabolic programming" or "imprinting" (Lucas, 1998; Lucas, 2000), has been used to explain associations between prenatal/neonatal environmental events, alterations in growth and development, and later pathophysiology (Barker et al., 1993; Seckl, 1998). For example, a study by Bell et al. (2003) demonstrated that early exposure to a diet high in fructose during the suckling-weaning transition may contribute to modest dyslipidemia later in life. The authors suggested that the changes observed in this study may increase the risk of cardiovascular disease in adulthood.

The ontogeny of the intestine may be influenced by early exposure to nutrients. The concept that intestinal sugar uptake was programmed was first suggested by Ginsburg and Heggeness (1968). In this study, rats were weaned onto a high carbohydrate diet (60%) or a carbohydrate free diet. Although the results suggested that intestinal sugar uptake was increased in the animals fed a carbohydrate-free diet, criticisms about the experimental design were noted by subsequent investigators like Karasov et al. (1985) who suggested that the results of the study were difficult to interpret. Karasov and and colleagues later studied the effect of feeding lactating dams either a no-carbohydrate, high-protein diet or a high-carbohydrate, maintenance protein diet and continuing to feed the offspring the same diet until they were eight weeks of age (Karasov et al., 1985). The high-carbohydrate diet produced irreversible increases in gut size and body weight, in conjunction with general increases in intestinal glucose transport. However, these changes in glucose uptake were reversible, since changing the diet abolished these alterations. Although this study failed to conclusively demonstrate the phenomenon of critical period programming, the investigators speculated that feeding the test diets during pregnancy may have permanent, irreversible effects on transport.

The role of dietary lipids in the programming of intestinal nutrient transport has been studied (Thomson and Keelan, 1987d; Thomson et al., 1989; Perin et al., 1997; Jarocka-Cyrta et al., 1998; Perin et al., 1999). Thomson and Keelan (1987d) demonstrated that feeding eight-week old rabbits a low cholesterol diet for two weeks reduced intestinal glucose uptake, and that this effect persisted for at least ten weeks. The response to diet depended on the duration of feeding, the age of the animals, and whether or not there was previous exposure to the diet. In this study, the effects on sugar transport were not explained by changes in food intake, body weight or intestinal weight. Furthermore, persistent changes were seen in the active transport of glucose, galactose, leucine and bile acids, while changes in the passive uptake of lipids were reversible.

Thomson et al. (1989) altered the ratio of polyunsaturated to saturated fatty acids in the diet of weanling rats. Diets enriched in saturated fatty acids increased hexose uptake, and these alterations were fast, progressive and irreversible. Feeding the same diets to pregnant and lactating rats resulted in similar increases in sugar uptake in their weanling offspring (Perin et al., 1997). Curiously, these changes were not seen in the suckling offspring, suggesting that the mechanisms responsible for adaptation may not be fully developed in these animals. Perin et al. (1999) confirmed that the weanling intestine was capable of adaptation, by continuing to feed the offspring of pregnant dams the same diet for three weeks post-weaning. Persistent alterations in sugar uptake were seen in response to variations in dietary lipids, once again emphasizing the importance of early exposure in the programming of intestinal nutrient transport. In addition to the differences between suckling and weanling offspring, the pattern of adaptation also appeared to differ between the jejunum and ileum.

Further studies went on to characterize the effect of diets enriched with arachadonic acid, docohexanoic acid and diets with different ratios of n6 to n3 fatty acids on intestinal nutrient transport (Jarocka-Cyrta et al., 1998). As in the previous study by Perin et al. (1997), these maternal diets critically influenced the ontogeny of the intestine, with many of the changes in transport being irreversible. Another important finding from many of these studies was that responsiveness to later dietary challenges depended on early experiences, once again emphasizing the importance of early dietary exposure.

Clearly, early diet influences transport later in life. But what other factors may influence intestinal function? In fact, several hormones and growth factors have been shown to cause precocious intestinal development, and to potentially stimulate intestinal growth and nutrient transport. However, it is not known if exposure to these factors has lasting effects on intestinal function.

2.4 AGING

Although each of us is familiar with inevitable age-related changes, the task of clearly defining the term is challenging. Agingis a multi-factorial process which includes both intrinsic and extrinsic factors. To further complicate matters, in humans the term can be considered from, including sociological, physiological, psychological and molecular perspectives.

While we tend to use the term "ontogeny" to describe development in early life, and "aging" to describe development (or sometimes presumed dysfunction) in later life, it is likely that a better concept is the development over the lifetime of the animal. Thus, "young" or "old" are descriptive terms describing a process over time. Aging then may be considered to be a continuumthat begins at conception and proceeds until death. The defintition of aging can be further refinedas "chronological age" according to the passage of time. Although advancing age is associated with increases in morbidity and mortality in general, this approach fails to consider the health of the individual. Determining a specific age at which an individual becomes "old" is arbitrary, and the concept of aging has changed over the centuries with humans now experiencing increased longevity and quality of life.

"Biological age" reflects the presence or absence of disease. Because there is not always a direct relationship between age and disease, this definition is considered to be a better marker of health status. The term "functional aging" has also been used to emphasize the limitations of defining health based on chronological age. This definition characterizes people based on what they can do in relation to others in society, but may also be used to characterize the level of functioning of organs and systems in the elderly. Finally, the concept of "successful aging" takes this idea one step further, and suggests that the aging process is variable, and may be characterized as a balance between gains and losses (Rowe and Kahn, 1997). The compression of morbidity and an enhanced quality of life are cornerstones of the concept of successful aging.

THEORIES OF AGING

A number of theories have been proposed to describe the process of aging. Longevity genes have been identified in many species, suggesting that aging may be at least partially under genetic control. In yeast, overproduction of the enzyme Sir2 prolongs the life of yeast grown under normal nutrient conditions (Lin et al., 2000). It has been suggested that increases in Sir2 (seen in response to caloric restriction or resveratrol, a polyphenol found in red wine) may increase gene silencing, and thereby result in greater genomic stability (Lin et al., 2000). Research undertaken in Drosophila has identified single gene mutations that extend life span. These include the gene Methuselah (mth), a secretion-type receptor that provides resistance to stress (Lin et al., 1998), and Indy (I'm Not Dead Yet), whose gene product is homologous to Kreb's cycle intermediates (Helfand et al., 2003; Marden et al., 2003). Using C. Elegans as a model, the gene daf2, an insulin/IGF-1 receptor homolog, has also been shown to affect lifespan (Kenyon et al., 1993). In humans, a genetic component to aging has also been suggested. Werner's syndrome, a disorder characterized by an apparent accelerated senescence, has been associated with a single gene locus on chromosome 8 (Yu et al., 1996). On the other hand, a genome wide scan of elderly subjects suggested that there is a locus on chromosome 4 that influences a person's genetic susceptibility to age well and to achieve exceptional longevity (Puca et al., 2001).

Cellular theories emphasize that the environment as well as intrinsic properties of the cell, often referred to as a "cellular clock", may limit survival. Pivitol research by Hayflick and Moorhead (1961) found that normal human fetal cell strains were limited to 40-60 doublings before they entered senescence. From this finding, they developed the concept of the "Hayflick limit" to explain thedetermination of longevity. From this early work, the concept of telomere shortening was then established as another mechanism of longevity determination. These repetitive DNA sequences found at the end of chromosomes are progressively depleted with age, and may represent a method by which cells enter senescence. This theory is not universally accepted, however, largely due to a lack of correlation between telomere length and life span in many animal species (Campisi, 2001).

Aging may be the consequence of oxidative damage. Oxidative damage to DNA, protein, carbohydrates and lipids contribute to degenerative diseases in aging, due to a disruption in cellular homeostasis. The activation of specific stress signalling pathways results in alterations in gene expression mediated by a variety of transcription factors including NF-κβ, p53, and heat shock transcription factor 1 (HSF1) (Finkel and Holbrook, 2000). While levels of antioxidants correlate with longevity in primates (Talmasoff et al., 1980; Cutler, 1984), it is not clear if antioxidant supplementation affects life span. It has been suggested, but not proven conclusively, that the success of calorie restricted diets in extending the lifespan of rodents is related to a reduction in free radical formation (Gredilla et al., 2001; Lass et al., 1998; Feuers et al., 1993). The role of insulin/IGF-1 signalling in the regulation of lifespan has been studied. The gene daf2, an insulin/IGF-1 receptor homolog has been shown to affect the lifespan of C. Elegans (Kenyon et al., 1993). Similarly, a related tyrosine kinase receptor, InR, regulates lifespan in Drosophila (Tatar et al., 2001).Holzenberger et al. (2003) demonstrated the importance of this pathway in mammals. In this study, heterozygous knockout mice (*Igf1r*$^{+/-}$) were used, as null mutants were not viable. These *Igf1r*$^{+/-}$ mice had IGF-1 receptor levels that were half of those seen in wild-type animals. These mice lived an average of 26% longer than did their wild-type littermates, without developing dwarfism, or showing adverse changes in physical activity, fertility or metabolism. This suggests that the link between insulin signaling and longevity seen in lower order organisms may also exist in mammals. Furthermore, the *Igf1r*$^{+/-}$ mice showed a greater resistance to oxidative stress, a known determinant of aging (Finkel and Holbrook, 2000). This lends support to the theory that oxidative stress plays an important role in the aging process.

In addition to increasing resistance to oxidative stress, insulin/IGF-1 signalling may affect aging via effects on Forkhead transcription factors, of the FOXO class. Overexpression of FOXO extends life span (Giannakou et al., 2004). Insulin/IGF-1 receptor binding, and subsequent activation of the PI3K/Akt pathway, results in the phosphorylation of Akt, which inactivates FOXO by sequestering it in the cytoplasm (Nijhout, 2003). This alters the effects of FOXO on resistance to stress, apoptosis and longevity, and provides another potential link between insulin/IGF-1 and aging.

Other theories of aging focus on neuro-endocrine changes, including reductions in the levels of the steroid hormone dehydroepiandrosterone (DHEA). Both animal and human studies have demonstrated that oral replacement of DHEA may prevent or reduce age-associated events such as cancer and cardiovascular disease, and may stimulate immune function (Schwartz and Pashko, 1995; Casson et al., 1996; Majewska, 1995; Morales et al., 1994, Khorram et al., 1997).

A SOCIETAL PERSPECTIVE

Seniors constitute the fastest growing segment of Canada's population. In fact, the proportion of seniors has risen from one in twenty in 1921, to one in eight in 2001. Within this group, the number of Canadians aged 85 or more is anticipated to increase substantially, up to 4% of the total population by the year 2041 (Health Canada, 2002). Women make up the majority of seniors, with gender differences becoming more pronounced in the oldest age groups.

The aging of the population may be thought of as a modern day success story. For the first time in history human beings have been afforded the opportunity to live an unprecedented number of years, with a reasonable quality of life. This accomplishment is not without challenges, as society struggles to adapt to a changing demographic, with a unique set of physiological, psychological, and social needs of the elderly themselves as well as their caregivers.

Several non-genetic factors may influence life expectancy, including improvements in sanitation and nutrition, as well as reductions in maternal mortality and the rates of infectious diseases (An Aging World:2001, U.S. Census Bureau). These changes, coupled with lower fertility rates, result in a changing demographic that presents society with the challenges of providing quality health care to an aging population, and facilitating the social, economic and community involvement of seniors.

Although most seniors rate their health as "good" or "very good", seniors are more likely to visit health care professionals, to take medication, and to be hospitalized when compared to their younger counterparts (Health Canada, 2002). Therefore, increases in this population and the associated increased health care utilization may place a burden on the system. Indeed, health expenditures for seniors in 2000-2001 represented 43% of total health care expenditures (Health Canada, 2002). Of course we recognize that these persons have contributed greatly to our society, and it is our responsibility to provide ready access to quality health care for these special persons, who musy be treated with respect and allowed to age with dignity.

The elderly are at a high risk for malnutrition, yet unfortunately it is often underdiagnosed (Tierney, 1996). Poor nutritional status is a key determinant of morbidity and mortality in the elderly (Kerstetter et al., 1992; Mowe et al., 1994; Sullivan et al., 1994; Payette et al., 1999). Because nutrition is a modifiable risk factor, attempts should be made to design preventative nutritional strategies aimed at improving the quality of life and consequently minimizing the use of health care resources.

Why are the elderly malnourished? There are a number of contributing factors including: (1) inadequate intake, attributed to a lack of appetite, or difficulty in preparing food; (2) psychological factors, including depression; (3) social factors, including isolation and low income; and lastly (4) physiological factors, such as reduced sense of smell and taste, drug-nutrient interactions and reductions in nutrient absorption (Payette, 2002). Sullivan et al. (1999) demonstrated that hospitalization was a risk factor for inadequate food intake in seniors, possibly due to the unattractive and monotonous food choices, or due to the side effects of drug therapies. Reduced food intake is generally accepted as the main cause of undernutrition in the geriatric population, and as such therapies should be aimed at increasing food intake. Many researchers also feel that malnutrition in the elderly is indicative of prevailing social conditions, and that therapies should be aimed at alleviating poverty, isolation and depression in this age group.

Because one of the factors that may contribute to malnutrition relates to age-associated alterations in the physiology of the gastrointestinal tract, this topic will be reviewed.

AGING AND THE GASTROINTESTINAL TRACT

The aging of the population, coupled with the potential impact on the health care system, has focused attention on the physiological processes associated with aging. Only with an increased understanding of the aging process can we work towards improving the quality of life for the elderly, and reducing disease morbidity in this population.

There are age-related alterations in the gastrointestinal tract but the difficulty lies in excluding concomitant pathological factors as the cause of these changes. Certainly with aging, conditions such as diabetes, pancreatic or liver disease, cancer, or drug-induced enteropathy will have potential adverse effects on the form and function of the intestine. It is necessary to exclude thesepathological factors, to consider the physiological changes that occur in the healthy elderly, and to understand how these factors influence the nutritional status of this population.

Dysphagia is more common in the elderly than in younger persons (Kawashima et al., 2004). Selective neurodegeneration may occur in the aging enteric nervous system (reviewed in Saffrey, 2004), and may contribute to gastrointestinal symptoms such as dysphagia, gastrointestinal reflux and constipation. Interestingly, caloric restriction in rodents can prevent the neuronal losses that occur with aging, suggesting that diet may influence gastrointestinal aging (Cowen et al., 2000). Alterations in esophageal motility may be due to reductions in the number of neurons in the myenteric plexus of elderly patients (Santer and Baker, 1988). While gastric motility may be impaired with aging (Moore et al., 1983; Wegener et al., 1988), small intestinal motility is unaffected (Fich et al., 1989; Altman, 1990; Madsen, 1992). Aging may affect the signal transduction pathways and cellular mechanisms controlling smooth muscle contraction, which may influence colonic motility and thereby contribute to the development ofconstipation (reviewed in Bitar and Patil, 2004).

The data regarding aging and gastric acid secretion is inconclusive, as early studies were likely confounded by the presence of *H. pylori* in some persons. Achlorhydria or hypochlorhydria may result from atropic gastritis, as a result of medications such as proton pump inhibitors, or as a result of H. pylori infection (Hurwitz et al., 1997; Pereira et al., 1998; Haruma et al., 2000). This reduction in gastric acidity may increase the risk of small bowel bacterial overgrowth, potentially leading to malabsorption (Saltzman and Russell, 1998). For example, McEvoy et al. (1983) found that 71% of patients in a general geriatric ward had bacterial overgrowth of the small intestine, while 11% were found to be malnourished. Indeed, bacterial overgrowth in older adults is associated with reduced body weight, which is paralleled by reduced intake of several micronutrients (Parlesak et al., 2003).

Although structural changes in the pancreas are seen with aging, no functional age-related alterations are seen using the fluorescein dilaurate test (Gullo et al., 1986). Some studies demonstrate reduced secretagogue-stimulated lipase, chymotrypsin and bicarbonate concentrations in pancreatic juice with aging (Laugier et al., 1991), other research suggests that there is little evidence of reduced pancreatic secretions with age, independent of other factors including the presence of disease and the effect of drugs (Dreiling et al., 1985).

There are age-related reductions in liver mass and blood flow, yet microscopic changes are subtle (Popper, 1986; Schmucker, 1988; Zoli et al., 1999). While structural and functional changes do not correlate well, there is evidence that liver function declines with age. For example, Cao et al. (2001) used microarrays to show that aging in mice is accompanied by changes in the expression of genes in the liver involved in inflammation, cellular stress and fibrosis, all of which are linked to age-related liver pathologies. Interestingly, caloric restriction in mice starting at weaning reversed the majority of the age-related changes, once again emphasizing the ability of the diet to influence the aging process.

Holt et al. (1984) looked at age-related changes in the intestinal morphology of Fischer 344 rats. Increases in villous width were noted throughout the small intestine, while increases in villous height were limited to the ileum. Other studies in rats have shown age-related losses in villous and enterocyte heights (Hohn et al., 1978). Age-related declines in mucosal surface area have also been reported in rabbit jejunum (Keelan et al., 1985b). Human studies generally show no changes in intestinal morphology, as determined from measurements of villous height, crypt depth, crypt-to-villous ratios and enterocyte size (Lipski et al., 1992; Corazza et al., 1986; Webster and Leeming, 1975). In contrast, Warren et al. (1978) showed a decrease in villous height with age. Martin et al. (1998) described histological changes that occur in aging mice: when old mice were compared to young mice, there were larger villi, a reduced number of crypts, and fewer villi and crypts per mm along the small intestine. These changes were most pronounced in the distal, as opposed to the proximal small intestine. Research has failed to find a clear association between intestinal morphology and nutrient uptake with aging. For example, despite reductions in mucosal surface area, aged rats demonstrated increases in the jejunal uptake of saturated fatty acids (Keelan et al., 1985). So, while it remains controversial as to whether or not aging is associated with morphological changes, even if such changes were to occur, the impact on nutrient uptake may not be clinically relevant.

Ciccocioppo et al. (2002) suggested that intestinal architecture is maintained with aging by increases in proliferation and differentiation rates. This agrees with work done by Corazza et al. (1998) that showed increased expression of proliferating cell nuclear antigen in older subjects when compared to their younger counterparts.

Age-related alterations in the abundance of brush border membrane(BBM) enzymes may also impact upon the digestion and subsequent absorption of nutrients. BBM lactase phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) activities fall with age in rats (Lee et al., 1997). Bacterial overgrowth, which is common in the elderly, may also negatively impact upon disaccharidase activity, and thereby possibly reducecarbohydrate absorption (Riepe et al., 1980).

Hollander and colleagues demonstrated that intestinal permeability to medium sized probes (mannitol, polyethylene glycol) increased in 28 month old rats when compared to 3 month old rats (Ma et al., 1992). However, a study done in humans shows that the lactulose:mannitol ratio was not different between young and old subjects, indicating that intestinal permeability to these sugars does not change significantly with age (Saltzman et al., 1995). ReseA study using breath hydrogen analysis following a carbohydrate meal showed evidence of malabsorption with aging. Elderly patients (ranging from 65-89 years, mean age,79 years) were compared to control subjects (ranging from 20-64, mean age,35 years). Significantly more subjects in the elderly group (7 out of 21) excreted excess H_2 when compared to controls (0 out of 19) (Feibusch and Holt, 1982). This suggests that there may be malabsorption of carbohydrates in the elderly. *In vitro* transport experiments using BBM vesicles also demonstrated a reduction in Na+-dependent D-glucose uptake in patients over the age of 70 (Vincenzini et al., 1989). In contrast, Wallis and co-workers (1993) did not find changes in Na+-dependent glucose transport in BBM vesicles isolated from duodenal biopsies from patients whose ages ranged from 55 to 91 years.

Experiments using rodent models of aging also demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985). Depending upon the intestinal site studied, a normal or increased absorptive capacity was also found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption. Ferraris et al. (1993) showed in aged mice a reduction in uptake and site density of the sodium dependent glucose transporter in the BBM, SGLT1. This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice. Our lab has recently investigated the effect of age on intestinal glucose uptake in Fischer 344 rats using the *in vitro* intestinal sheet method (Drozdowski et al., 2003). Glucose uptake was reduced in 9 month old and 24 month old rats when compared to 1 month old animals. When changes in mucosal surface area were accounted for, only ileal glucose uptake was reduced in the older animals. These age-associated changes in glucose uptake were not explained by alterations in the abundance of SGLT1, GLUT2 or Na^+K^+-ATPase abundance.

The variations in the results from human, rat and mouse studies may be due to the differences in the methodologies that were used. While some investigators studied uptake using BBM vesicles (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985; Vincenzini et al., 1989; Wallis et al., 1993), others used everted intestinal rings (Darmenton et al, 1989; Ferraris and Vinnekota, 1993; Thompson et al., 1988) or intestinal sheets (Drozdowski et al., 2003). As well, the method of expressing results may contribute to the differences between studies. Uptake is often expressed on the basis of intestinal weight, and does not taken into account any potential age-associated changes in mucosal weight or surface area. The strain and ages of the animals, and the site of the intestine used also differ between studies, and may explain the variability in the results.

The uptake of fructose has been studied in aging mice. Ferraris and Vinnekota (1993) showed that D-fructose uptake per milligram of tissue was higher in the jejunum of young as compared to old animals. Adaptive increases in uptake, in response to increases in carbohydrate levels, were blunted in these mice, and were restricted to more proximal regions of the small intestine

While it is reasonable to speculate that the complexity of lipid absorption may make it susceptible to the effects of aging, experimental findings do not consistently support this notion. While a number of animal studies demonstrate reduced *in vitro* lipid absorption with aging (Thomson, 1980; Flores et al., 1989), others have shown increases in lipid absorption in aged rats using an *in vivo* perfusion model (Hollander and Dadufalza, 1983). Aging is associated with a decrease in the thickness and resistance of the unstirred water layer (Thomson, 1980), which could partially explain the finding of increased absorption with aging in the *in vivo* model.

Early work using human subjects demonstrated reductions in lipid absorption with age (Becker et al., 1950). There also appears to be reduced intestinal absorption of bile acids with age (Salemans et al., 1993), although it is not clear if this negatively impacts lipid absorption in the elderly. When healthy elderly human subjects were studied, however, no correlation between age and 72 hour fecal fat excretion was found (Arora et al., 1989). After considering the results of all of these studies, Holt (2001) suggested that no important changes in lipid absorption with aging have been described.

More recently, a study by Woudstra et al. (2004) showed that the ileal uptake of several fatty acids including 16:0, 18:0, 18:1 and 18:2, was reduced in 24 month old rats, when compared to 1 month old animals. However, when mucosal surface area was considered these differences disappeared, suggesting that the age-related changes in lipid uptake were largely due to non-specific reductions in intestinal surface area.

MODIFICATION OF THE AGE-ASSOCIATED DECLINE IN INTESTINAL ABSORPTIVE FUNCTION

In adult rats, a diet enriched with saturated fatty acids (SFA) results in increased intestinal sugar uptake when compared to an isocaloric diet enriched with polyunsaturated fatty acids (PUFA) (Thomson et al., 1987b; Thomson et al., 1988; Thomson et al., 1991; Thiesen et al., 2003d). Similarly, Vine et al. (2002) studied the effect of various fatty acids on the passive and active transport properties of rat jejunum, and found that an SFA diet increased Na^+-dependent glucose uptake when compared to a diet enriched with n6 PUFA. Of importance, Woudstra et al. (2004) showed that the intestinal response to dietary lipids may differ with age. The mechanism responsible for this age-related alteration in adaptation to dietary lipids is not known. But clearly, the results of adaptive studies in young rats do not necessarily apply to older animals.

2.5 HORMONES AND GROWTH FACTORS

This review will focus first on insulin-like growth factors, epidermal and transforming growth factors, thyroid hormones and glucocorticosteroids. The second section will detail the effects of glucagon-like peptide 2 (GLP-2) on intestinal absorption and function and the potential for an additive effect of GLP-2 and glucocorticosteroids.

INSULIN-LIKE GROWTH FACTORS (IGF-I, IGF-II)

The insulin-like growth factor family consists of insulin, insulin-like growth factor I (IGF-1), and insulin-like growth factor II (IGF-II). The liver is the major site of synthesis for IGF-I, but it is also synthesized in other tissues including the gastrointestinal tract (Jones and Clemmons, 1995; Yang and Ney, 1994). IGF-I and IGF-II are found in human milk (Donovan et al., 1991), and these peptides are members of a complex mixture of growth factors that the neonate is exposed to during the suckling period.

Intestinal development may involve IGF-I and IGF-II (Beck et al., 1987, Dvorak et al., 1996, Orlowski et al., 1990) IGF-I receptor (UGF-1R) (Schober et al., 1990, Young et al., 1990) expression is detectable in the fetus and the neonatal animal. In humans, IGF-I, IGF-II and IGF-IR are also present in fetal intestine (Hormi and Lehy, 1994; Menard et al., 1991; Han et al., 1988).

Intestinal cells proliferate in vitro when exposed to IGF-I (Park et al., 1990; Conteas et al., 1989; Kurokowa et al., 1987; Duncan et al., 1994). Transgenic mice that overexpress IGF-I demonstrate increased small intestinal growth (Ohneda et al., 1997), particularly in the muscle layers of the distal small intestine and large intestine (Wang et al., 1997). IGF-I receptor gene knockout animals have fetal growth retardation, but distinctive effects on the gastrointestinal system have not been found (Liu et al., 1993). This may indicate the presence of redundant systems in the gastrointestinal tract that compensate for the lack of IGF-1.

In vivo studies show variable responses depending on the dose of IGF-I that is used. Pharmacological doses of IGF-1, given orally to colostrum-deprived 5 day old piglets, increase electrolyte and nutrient absorption (Alexander and Carey, 1999). Neonatal pigs fed formula supplemented with IGF-1 from birth to 4 days of age had increased mucosal growth (Burrin et al., 1996). Parenterally fed 1 day old piglets given enteral IGF-1 had increased brush border membrane (BBM) disaccharidase levels (Park et al., 1999). Houle et al. (1997) administered IGF-I orally to neonatal rats, and demonstrated increases in mucosal growth and BBM enzymes. Similarly, Burrin et al. (1996) showed increases in intestinal weight, protein, and DNA in neonatal pigs fed orogastrically. Alexander and Carey (1999) examined the effect of oral IGF-I on neonatal piglet intestine, and found increases in D-glucose uptake in everted jejunal sleeves, independent of the intestinal mass or surface area. Lane et al. (2002) used RT-PCR and laser scanning confocal microscopy to show that intragastric IGF-I or IGF-II increased the expression of the glucose transporters SGLT1 and GLUT2 in suckling rat intestine.

The mechanisms responsible for increased jejunal transport rates observed in tissues treated with orally administered insulin-like growth factor-I (IGF-I) may include the increases in Na^+K^+-ATPase seen with IGF-1 treatment. The PI3-kinase pathway may also be important, as preincubation with a PI3-kinase inhibitor abolishes the effects of IGF-1 on ion and nutrient transport (Alexander and Carey, 2001).

Although pharmacological doses of IGF-I have clear effects on the neonatal intestine, studies using physiological doses show only limited effects. For example, recent studies with both neonatal pigs (Houle et al., 1997, Xu et al., 1994) and calves (Baumrucker et al., 1994) have shown that the oral administration of physiological concentrations of IGF-I in formula results in measurable increases in crypt cell proliferation, but there are no demonstrable increases in intestinal mucosal mass or length.

EPIDERMAL GROWTH FACTOR (EGF) and TRANSFORMING GROWTH FACTOR-α (TGF-α)

EGF and TGF-α are two ligands of the EGF receptor that have been postulated to play a role in intestinal ontogeny. Both peptides are present in human breast milk, with TGF-α at a 5-10-fold lower concentration than EGF (Okada, 1991). Transforming growth factorβ (TGFβ) is a family of growth factors, with the three isoforms (TGFβ1, TGFβ2, TGFβ3) having nearly identical biological activities. TGFβ1 and TGFβ2 are present in human milk (Saito et al., 1993), and mRNA and protein corresponding to the three TGFβ isoforms are found in the intestinal mucosa (Thomas et al., 1992, Barnard et al., 1989, Barnard et al., 1993). Small intestinal development, however, is not severely impaired by the targeted disruption of the TGFβ gene (Dickson, 1995)

EGF and TGF-α have been isolated in human 15-20 week gestation fetal intestine, with TGF-α levels as much as 10 times higher than EGF (Miettinen et al., 1989). EGF receptor expression is detectable throughout the GI tract in the human fetus and neonate. The EGF receptor is developmentally regulated with expression being low during the human suckling period, and in rodents, unlike humans, EGF receptor expression is delayed until weaning (Chailler and Menard, 1999).

Oral EGF administration augments gut growth and functional development in neonatal rats and pigs (Berseth, 1987, Jaeger et al., 1990; Zijlstra et al., 1994). Gene targeting studies have demonstrated that disrupting the expression of the EGF receptor in mice resulted in impaired intestinal development, and death associated with a necrotizing enterocolitis-like disorder (Miettinen et al. 1995).

The postnatal development of intestinal transport and the physical composition of the BBM were examined in New Zealand White rabbits receiving EGF (40 micrograms/kg/d) either intraperitoneally or orogastrically from day 3 to day 17 of life. Intestinal water, Na^+ and glucose absorption expressed per cm of intestine were significantly increased in animals receiving EGF by either route. Increased absorption induced by orogastric EGF appeared to be secondary to mucosal hyperplasia. The BBM isolated from EGF-treated animals was significantly more fluid than that of controls. These results suggest that EGF modulates the development of transport function during the postnatal period both by stimulating mucosal growth and by inducing specific transport processes (Opleta-Madsen et al., 1991).

Experimental necrotizing enterocolitis (NEC) was induced by exposure of newborn animals to asphyxia and cold stress. Newborn rats were artificially fed either with growth factor-free rat milk substitute or the same formula supplemented with 500 ng/ml of EGF (Dvorak et al., 2002). EGF supplementation of formula reduced the incidence and severity of NEC, as assessed by gross and histological scoring of the ileum. This finding suggests a potential therapeutic approach for the prevention and treatment of NEC.

Erythopoietin (Epo) is present in breast milk (Kling et al., 1998), and receptors for Epo are present on intestinal cells (Juul et al., 1999). Modest changes in small intestinal length and surface area are observed following subcutaneous Epo injections in artificially fed rat pups.

Hepatocyte growth factor (HGF) is also present in breast milk, and both HGF and its receptor are present in fetal intestinal tissue (Kermorgant et al., 1997, Wang et al., 1994). Keratinocyte growth factor (KGF) promotes proliferation and up-regulates

SI expression in fetal human small intestine explant culture (Chailler et al., 2000). It remains to be seen if HGF or KGF plays a physiological role in postnatal intestinal development.

The thyroid hormones triiodothyronine (T3) and thyroxine (T4) have been implicated in intestinal morphogenesis, based on T3Rα knockout studies (Shi, 1995). The effect of thyroid hormones on the ontogeny of BBM enzymes has also been examined. An in vivo study of lactase phlorizin hydrolase (LPH) catalytic activity, synthesis, and degradation was performed in propylthiouracil-induced hypothyroid rat pups, hypothyroid pups injected with thyroxine, and normally weaned rats. T4 regulates LPH ontogeny by posttranslational mechanisms that include altered processing and increased degradation of the BBM lactase enzyme (Liu et al., 1992).Monteiro et al. (1999) examined the role of T4 on the precocious enhancement of GLUT5 in weanling rats. Rat pups were made hypothyroid by giving the dam 0.01% propylthiouracil in the drinking water from day 18 of gestation. The hypothyroid pups and age-matched euthyroid control pups were then fed high-fructose solutions by gavage, twice a day starting at 17 days of age for 3 days. Although serum T4 levels were five times lower in the hypothyroid pups, the mRNA levels for the BBM fructose transporter (GLUT5) increased in euthyroid and hypothyroid pups fed high fructose. This result paralleled the increase in fructose uptake. This suggests that during weaning, dietary fructose can precociously enhance intestinal fructose uptake and GLUT5 mRNA expression, independent of developmental increases in serum T4 levels.

GLUCOCORTICOSTEROIDS

Biochemistry

Steroids can be divided into two categories: glucocorticosteroids (GC), if potency is based on liver glycogen deposition, and mineralocorticoids, if potency is based on sodium retention. The hormones that are secreted in significant amounts are cortisol, corticosterone, and aldosterone. In humans, the major naturally occurring steroid is cortisol, while in rodents the main steroid is corticosterone.

Steroids are synthesized from cholesterol in the inner mitochondrial membrane of the adrenal glands, through a series of reactions including hydroxylations. The release of cortisol from the adrenal glands is regulated by the hypothalamic-pituitary-adrenal axis, and involves the secretion of corticotropin-releasing hormone from the hypothalamus, and the subsequent secretion of adrenocorticotropin hormone (ACTH) from the pituitary gland. The rate-limiting step in steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane by sterol carrier protein 2 (SCP2), steroidogenesis activator protein polypeptide (SAP), peripheral benzodiazepine receptor (PBR), and steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996). Because corticosteroids are not stored in adrenal tissues, the rate of secretion equals the rate of biosynthesis (Haynes and Murad, 1985; Haynes and Larner, 1975; Simpson and Mason, 1979; Bowman and Rand, 1980).

GC are classified as lipids because they are more soluble in organic solvents than aqueous solvents. Their solubility is affected by the presence of hydroxyl or carbonyl groups (Myles and Daly, 1974). GC enter cells by diffusion across the plasma membrane. About 90% of circulating cortisol is reversibly bound to a plasma protein, the corticosteroid binding globulin (CBG) (Haynes and Murad, 1985; Myles and Daly, 1974; Bowman and Rand, 1980). CBG is a high affinity, low capacity plasma protein, while albumin has a low affinity and high capacity to bind steroids (Haynes and Murad, 1985; Myles and Daly, 1974; Bowman and Rand, 1980; Girdwood and Petrie, 1987). The bioavailablility of GC in specific tissues is determined by the presence of tissue specific metabolizing enzymes (11-β-hydroxysteroid dehydrogenase (11β-HSD)), CBG levels, the presence of efflux proteins (such as multi-drug resistance protein 1, MDR1), and the expression of the glucocorticosteroid receptors (GR). Other levels of regulation include variations in the receptor protein (isoforms, polymorphisms), alternative receptor dimerization, co-chaperones, levels of hsp, and posttranslational modifications (reviewed in De Bosscher et al. (2003).

Approximately 70% of corticosteroid metabolism occurs in the liver, and involves the cytochrome P450 system (Brattsand, 1990). Intestinal metabolism is also important, as the intestinal sites of cortisol and cortisone metabolism in humans are saturated before the hepatic sites (Barr et al., 1984). The majority of corticosteroids are excreted in the urine, although small amounts are detectable in fecal, biliary and pulmonary CO_2 excretions (Haynes and Murad, 1985; Haynes and Larner, 1975; Sutherland, 1970).

Glucocorticosteroid receptors

Glucocorticosteroid receptors (GR) are 94 kDa proteins found in the cytoplasm of cells from many tissues. When ligand binding occurs, an inhibitory protein is released, the receptor dimerizes, hyperphosphorylation occurs (Hu et al., 1994, Orti et al., 1992; Bodwell et al., 1993), and a DNA binding site is exposed (Figure 8). This conformational change, which may unmask nuclear localization signals, allows the GR to translocate to the nucleus. The GR may affect gene transcription and subsequent protein synthesis by interacting with specific nuclear binding sites (referred to as glucocorticosteroid response elements, GRE). This causes either stimulation or, less frequently, inhibition of transcription. Also, the binding of the GR homodimer to the GRE may induce rearrangement of the chromatin, allowing other transcription factors to bind to previously inaccessible DNA (Li and Rosen, 1994).

The GR may also interact with other transcription factors, such as AP-1, in which case transcription is commonly inhibited (Jonat et al., 1990; Yang-Yen et al., 1990). There are several other transcription factors that have been linked to the GR, including the p65 subunit of NFκB (Ray and Prefontaine, 1994; Caldenhoven et al., 1995).

The unliganded GR may be associated with heat shock proteins (hsp), including hsp56, hsp70 and hsp90. There may be constant cycles of dissociation/reassociation of these components, as well as constant bi-directional shuttling of GR between the cytosol and the nucleus (Pratt, 1993; Madan and DeFranco, 1993). Furthermore, the action of hsp70 and hsp90 may be further regulated, either positively or negatively, by co-chaperones (Pratt and Toft, 1997; Defranco and Csermely, 2000; Cato and Mink, 2001; Davies et al., 2002). These proteins may also be involved in recruiting transport proteins to the receptor complex, regulating hormone binding to the receptor, and modulating the regulatory function of

GR by disassembling transcription complexes (Cato and Mink, 2001; Davies et al., 2002;Freeman and Yamamoto, 2002; Morimoto, 2002).

Several other factors may influence GR-mediated transcriptional activation. For example, it has been suggested that competition between transcription factors for limited coactivator molecules may lead to gene repression (Gerritsen et al., 1997; Sheppard et al., 1999). Alternatively, cofactor effects may be restricted to designated compartments, and dynamic cofactor modules may hit promoters in a cyclic way during transcription (Tansey, 2001; Kino and Chrousos, 2003; Freeman and Yamamoto, 2002; Morimoto, 2002). In addition to acetylation, methylation and phosphorylation of histones may influence transcriptional control (Cheung et al., 2000; Strahl and Allis, 2000; Santos-Rosa et al., 2002), as also could phosphorylation of the GR (Rochette-Egly, 2003).

Some of the effects of GC occur too rapidly to be explained at the genomic level. Distinct GR forms or a cytosolic subset of GR may interact with signal transduction pathways which are usually associated with membrane receptor signalling events (Simoncini et al., 2000). Extensive crosstalk between steroid- and growth factor-stimulated signalling pathways occurs, and may impact the functioning of steroid receptors and genes that are regulated by steroids (reviewed in Lange, 2004).

A number of synthetic GC have been developed with increased potency when compared to the naturally occurring substances. The increased potency, however, comes with an increased potential for adverse effects. Dex is a synthetic steroid with a potency ~25 times that of cortisol (Ganong, 1997). The high potency of Dex is attributed to its long half life and high affinity for the GR (Ganong, 1997). Dex is commonly used in the research setting due to its' unique properties. The synthetic Dex differs from the naturally occurring cortisol due to the addition of a double bond between carbon 1 and 2, fluorination of the 9α position; and the addition of a methyl group (Figure 9). Therefore, while most GC in the plasma are bound to the CBG, Dex circulates freely, with blood concentrations unaffected by changes in CBG levels. This is particularily important in studies of development, as CBGs are known to be developmentally regulated (D'Agostino and Henning, 1981). The placental enzyme

11-β hydroxysteroid dehydrogenase type-2 (11β-HSD$_2$) protects the fetus by converting maternal cortisol into inactive cortisone, due to its dehydrogenase activity (Godfrey, 2002). However, the unique structure of Dex allows it to cross the placenta by escaping deactivation by 11β-HSD$_2$.

What is the effect of GC on the fetal intestine? Although some studies have shown that GC are not important in fetal development (Solomon et al., 2001; Gartner et al., 2002), not all researchers would agree with the suggestion that the fetus is unresponsiveness to GC. For example, Buchmiller and colleagues (1994) showed that maternal Dex administration produced increases in small intestinal length and a trend towards an increase in LPH and maltase activity in fetal rabbits. The everted sleeve technique was used to demonstrate increases in glucose and proline transport in fetal rabbits exposed to Dex. When the fetal rabbits were subdivided into categories based on uterine position, runt fetuses exposed to Dex exhibited high nutrient uptake rates, surpassing the rates of the favored fetuses. This suggests that the runt intestine may be more responsive to steroid treatment, an observation with interesting implications regarding the potential clinical treatment of low birth weight or growth retarded infants.

The effects of GC on fetal intestine were also examined in a rabbit gastroschisis model. Gastroschisis is a herniation (displacement) of the intestines through a defect on one side of the umbilical cord. Intra-amniotic Dex (0.2 μg/g/d) infusion enhanced intestinal disaccharidase activity and glucose uptake in fetuses with experimentally induced herniation of the small intestine into the amniotic cavity (Guo et al., 1995). While this study looks at a specific congenital dysfunction, it clearly demonstrates that the fetal intestine is capable of responding to GC.

What is the effect of GC on the intestine of the neonate? Nanthakumar et al. (2005) used human intestinal xenografts to characterize the response of the developing intestine to GC. Responsiveness was determined by lactase activity and cytokine induction after a proinflammatory stimulus. Immature transplants (20 weeks) responded to GC, but that this effect was lost in the mature (30 weeks) transplants. This suggests that in humans there may also be a brief period of responsiveness in utero to GC.

Costalos et al. (2003) showed that in humans, maternal Dex administration increased fetal and neonatal plasma gastrin concentrations, while motilin was increased only in neonates, and vasointestinal peptide concentrations were unchanged. The authors speculate that the effects of GC on the gastrointestinal tract may be at least partially mediated by their actions on other GI hormones. Clearly, there is contradictory evidence regarding the ability of the fetus to repond to steroids. The discrepancies may be due to the use of different indicators (trehalase, SI, nutrient uptake, morphology) of GC responsiveness. Maternal DEX administration may have a direct effect on the fetus, as DEX is known to cross the placenta (Reece et al., 1995). However, it is also possible that the maternal administration of DEX has an indirect effect on the fetus, through the modulation of other factors such as cytokines, hormones or growth factors.

The effect of GC on prenatal intestinal maturation was investigated by Gartner et al. (2002), using mice lacking a functional glucocorticoid receptor (GR-/-). Although a temporal association exists between the prenatal GC surge and corresponding intestinal development, the results of this study suggest that steroids are not mandatory for prenatal intestinal maturation. Histological examination of 18 day old fetal mice failed to demonstrate any effects of the GR-/- phenotype on intestinal morphology or on measures of functional development (including enterocyte, Goblet and enteroendocrine cell numbers). LPH activity (as measured by X-gal staining) and the number of proliferating cells (as assessed by KI-67 staining) were also unaffected by the absence of GR. Thus, the rodent intestine appears to pass through a period of unresponsiveness to GC during fetal life, prior to becoming responsive in the postnatal period.

The role of GC in postnatal development has been well characterized. Indeed, adrenalectomy during the suckling period retards the functional maturation of the small intestine, while exogenous administration of GC may cause precocious maturation (Moog, 1971; Galand, 1989; Solomon et al., 2001). The mechanisms by which GC influence maturation are not completely understood. There may be direct as well as indirect effects of GC due to alterations in other factors. For example, Schaeffer et al. (2000) injected hydrocortisone (50μg/g, sc) into 11 day old rats. They showed that the precocious induction of the BBM enzyme sucrase isomaltase (SI) paralleled decreases in TGF-β and the cytokine IL-1β, and increases in TNF-α. This finding suggests that cytokine levels may mediate the effects of GC on postnatal intestinal maturation.

The development of GC responsiveness was characterized in more detail by Solomon et al. (2001). Using trehalase as a marker of intestinal maturation, they showed three phases of responsiveness to GC in mice: 1) a prenatal phase during which time Dexamethasone (DEX) did not induce maturation; 2) an early postnatal phase (first 2 postnatal weeks) of moderate responsiveness; and 3) a subsequent phase of increasing responsiveness in the third postnatal week. Postnatal increases in responsiveness were not paralleled by increases in GR abundance, but were associated with elevated circulating T4 concentrations. This observation fits with previous work demonstrating a synergistic effect of GC and T4 on BBM enzyme expression (Yeh et al., 1991; Leeper et al., 1998).Nanthakumar and Henning (1993) also previously demonstrated three post-natal phases of responsiveness to GC. Based on the effects on SI mRNA and activity in rats, they identified 1) an early phase (day 10) when activation of the gene occurs; 2) a late phase (day 16) when changes in cell kinetics are observed; and 3) a loss of responsiveness (day 18) to GC. Although the exact timing of the various phases is not consistent between studies, it is clear that steroids have maximal effects in the early postnatal period.

Does the adult small intestine remain responsive to steroids? Foligne et al. (2001) attempted to answer this question. Three month old Sprague Dawley rats underwent bilateral adrenalectomies, and were sacrificed 10 days after surgery. Adrenalectomy modified maturation and differentiation, particularily in the proximal small intestine. A partial atrophy and disorganization of villous architecture was noted, coupled with decreases in crypt cell proliferation and increases in apoptotoc cells in the upper villous region. LPH and SI were increased by adrenalectomy, while aminopeptidase N and intestinal alkaline phosphatase activities were reduced. These results indicate that the adrenal glands and GC play an important role in the trophic status of the small intestine in adult rats.

GC are used to treat various disorders including rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, allergies and asthma due to their anti-inflammatory effects (Xu et al., 2001). Both NFκB and AP-1 are crucial for the induction of many genes involved in inflammation. GC may interfere with the transcriptional activity of these and other factors. Alternatively, GC may also interact with a negative GRE (nGRE) and inhibit transcription. Although the anti-unflammatory effects of GC may be due to negative modulation of pro-inflammatory factors (transrepression), the side effects may be the result of transactivation (Nishioka and Reinberg, 2001). This has created interest in developing "dissociating steroids", in which the desireable transrepression effect is separated from undesireable transactivation effect.

Glucocorticoid resistance may occur as GC reduce GR expression (Okret et al., 1986). This correlates with activation of MAPK and NFκB. Combining GCs with MAPK inhibitors have shown therapeutic efficacy (Bantel et al., 2002; Irusen et al., 2002).

The importance of GC is clear from the clinical symptoms associated with a deficieny or excess of the hormones. Cortisol deficiency (Addison's disease) is characterized by postural hypotension, weight loss and hypoglycemia, while GC excess (Cushing's syndrome) is characterized by hypertension, central obesity and glucose intolerance (Andrews and Walker, 1999). There are many other adverse effects of the therapeutic administration of GC (Ganong, 1997).

GC may also be used to accelerate lung maturation and surfactant production in premature infants, although Dex is not recommended as it is associated with impaired growth and neurodevelopmental delay (Joint Statement, Canadian Pediatric Society, 2002).

GC administration reduces the incidence of NEC. A prospective experiment looking at prenatal or postnatal steroid use demonstrated a reduction in NEC in neonates (Halac et al., 1990). Animal models of Dex administration demonstrate increased mucosal maturation, with increased enterocyte and goblet cells, coupled with a thinning of the muscularis (Gordon et al., 1999). Unfortunately, early postnatal administration of Dex is associated with the development of focal small bowel perforation (Gordon et al., 1999; Stark et al., 2001). The mechanism associated with the differential effect of GC on intestinal mucosa and muscularis is not fully understood. Although in vitro work on intestinal smooth muscle cells suggests that Dex alters the expression of collagen and other basement membrane elements (Walsh et al., 1987; Graham et al., 1995), in vivo remodelling of the extracellular matrix by Dex has not been shown.

The tissue specific effects of GC may be the result of a redistribution of growth factors. Dex administration in newborn mice alters IGF-1 immunolocalization, with increased protein detected in the mucosa, and reduced levels in the muscularis (Gordon et al., 2001). In situ hybridization analyses for IGF-I transcripts showed no differences in localization between the groups. In addition to its' effects on IGF-1 immunolocalization, Dex (i.p 1μg/g, once daily) altered IGF-1 binding protein composition in the mucosa in newborn mice. This alteration may be responsible for drawing IGF-1 from the mesenchyme to the mucosa, and subsequently influencing intestinal maturation (Gordon et al., 2002).

Further support for the involvement of the IGF-1 system in NEC comes from the work of Burrin et al. (1999), who showed that in addition to decreases in circulating IGF-1 and alterations in IGF-1 binding proteins, neonatal pigs treated with Dex (subcutaneous, 1 mg/kg, for 7 days) had increased IGF-1 receptor mRNA abundance. This increase was only observed in the stomach and ileum, with no effect seen in the jejunum. This corresponds well with the authors' observation that the effects of Dex are most pronounced in the ileum. The catabolic effects of Dex were also characterized in this study: small intestinal growth, particularily in the ileum, was inhibited due to increases in protein degradation, without significantly affecting protein synthesis.

GC have well documented effects on the ontogeny of BBM hydrolases. Adrenalectomy delays enzyme maturation during the third post-natal week, while exogenous GC administration may induce the precocious appearance of SI activity (reviewed in Henning et al., 1994). In adult animals, however, disaccharidase activities in the small intestine are unaffected by adrenalectomy or by GC administration (Deren et al., 1967). The administration of DEX in the neonatal period increases the intestinal uptake of sugars (Drozdowski et al., unpublished observations, 2005).

There may be a syngeristic interaction between GC and T4 on BBM enzyme maturation. This was thought to involve the ability of T4 to increase corticosteroid-binding globulin, which subsequently reduces the clearance of hydrocortisone (Smith and Hammond, 1992). However, work by McDonald and Henning (1992) demonstrated syngergism between Dex and T4. This indicates that an additional mechanism must be involved, since Dex does not bind to the CBG. In this study, animals received daily injections of subcutaneous T4 (130 pmol/gram body weight) and a non-saturating dose of Dex (0.01 μg/gram body weight) from post-natal days 5 to12. The hormones synergistically increased jejunal SI, as well as ileal and duodenal alkaline phosphatase, and decreased ileal β-galactosidase activity, but did not affect jejunal or ileal LPH activity. When administered alone, T4 did not affect intestinal maturation, and Dex only partially stimulated maturation, when compared to the combination of hormones. The authors concluded that enzymes that rise post-natally responded to treatment with the combination of T$ and DEX, while enzymes that decline post-natally show a mixed response.

In suckling rodents, GC regulate the expression of BBM enzymes such as SI-isomaltase and trehalase (Henning et al., 1994; Leeper and Henning, 1990; Oesterreicher et al., 1998). However, following GC administration, it takes ~12-24 hours to observe increases in the transcription of these genes. This delayed time-course suggests the involvement of secondary response genes (Dean and Sanders, 1996). Oesterreicher and Henning (2004) identified a region of the trehalase promoter with potential binding sites for several transcription factors. Electomobility shift assays were performed using oligonucleotides from this region, as well as nuclear extracts from jejunum of 8 day old control or Dex-treated (1μg/g body wt) mice. They found that Dex stimulated expression of GATA-4 and GATA-6 proteins. These transcription factors are recognized as being important regulators of intestinal gene expression, and may interact with other transcription factors including those from the Sp family, Cdx, HNF-1 and HNF-4 (Boudreau et al., 2002; Divine et al., 2003; Dusing et al., 2003; Krasinski et al., 2001). Indeed, GATA factors may allow cooperating transcription factors to bind to the DNA by altering the chromatin structure (Cirillo et al., 2002). Although this study failed to prove that the induction of GATA factors leads to transcriptional activation of the BBM hydrolases, it does increase our understanding of possible mediators of GC effects.

Other molecular signals that may be responsible for the ontogenic changes in intestinal gene expression include a group of transcription factors called CCAAT/enhancer binding proteins (C/EBPs). In an attempt to determine regulatory mechanisms involved in the expression of the C/EBP alpha, beta and delta isoforms, Boudreau et al. (1996) examined their expression in response to GC in the rat intestinal epithelial crypt-derived cell line IEC-6, using Northern blot, transcription run-on assays, indirect immunofluorescence, Western blot, and electrophoretic mobility shift assays. Whereas C/EBP alpha expression was not regulated by GC, C/EBP beta and delta mRNA and protein levels were rapidly induced. Moreover, C/EBP beta- and delta-containing DNA binding complexes were increased by GC as determined by supershift assays, in contrast to C/EBP alpha containing complexes. Immunofluorescence studies showed cytoplasmic and nuclear localization for C/EBP alpha. This is in contrast to a restricted nuclear localization for both C/EBP beta and C/EBP delta. Differential regulation by GC, as well as the different localization of three C/EBP isoforms, suggests a role for this class of transcription factors in the control of gene expression in intestinal epithelial cells.

Mechanisms of Interaction

The mechanisms by which GC exert their effects may involve several other factors. Boudreau et al. (1999) showed that Dex increases c-fos and c-jun, and increased AP-1 DNA-binding capacity in IEC-6 cells. Ras transformation repressed the growth-inhibitory properties of Dex, and inhibited the induction of c-fos protein and mRNAsuggests that Ras negatively modulates the response of intestinal epithelial cells to GC. Thiesen et al (2003b) have shown that early response genes may be involved in the effect of GC in the enhancement of intestinal nutrient absorption that occurs following intestinal resection. This illustrates further the cross-talk that occurs between GC and intracellular signalling pathways.

GC may also exert their effects by influencing cellular proliferation, differentiation and apoptosis. Foligne et al. (2001) investigated the effects of 10-day bilateral adrenalectomy on morphometry, proliferation and apoptosis in the small intestine of 3 month old Sprague-Dawley rats. Adrenalectomy led to partial atrophy and disorganization of the epithelium, with an increased number of goblet and Paneth cells. A reduction of crypt cell proliferation was paralleled by a marked increase in apoptosis. Several other studies suggest that steroids may increase apoptosis. In vitro studies using rat jejunal epithelial cells (IEC-6) cells show that the locally acting budesonide increases apoptosis, while in vivo studies show that steroids increase apoptosis in intraepithelial lymphocytes (Murosaki et al., 1997; Ruiz-Santana et al., 2001).

There are conflicting reports on the effect of GC on intestinal proliferation. Low to medium doses (10^{-6}-10^{-11}) increase proliferation in IEC cells, while high doses (10^{-5}) inhibit proliferation (Goke et al., 2002). Other studies suggest that the effect of steroids on proliferation depends on the developmental stage of the animal (Sauter et al., 2004), Similarly, the location of the cells along the crypt-villous axis is an important factor as Dex reduced proliferation in the upper but not the lower crypt of fetal mouse dudodenal explants (Beaulieu et al., 1987). This points to an important role of the adrenal glands and glucocorticoids in the trophic status of the adult small intestinal mucosa. These results also highlight the fact that the atrophy associated with Gc is associated with a reduction in proliferation coupled with an increase in apoptosis. It is possible that some of the negative effects of GC could be reduced or prevented by the administration of a trophic agent.

Quaroni et al. (1999) found that exposing IEC cells to GC cause growth arrest, the formation of tight junctions, the appearance of tall slender microvilli, reorganization of the ER and Golgi, and decreased cdk6 as well as p^{27kip1}protein. These results are consistent with the activation of multiple genes important in the functioning of absorptive villous cells, but likely not involved in the induction of cell differentiation.

The role of the mesenchyme in mediating the effects of GR was studied by Simo et al. (1992). GC treatment of mesenchyme-derived cell populations resulted in an accumulation of laminin at the cell surface, accompanied by enhanced expression of BBM enzymes. This effect was abolished by anti-laminin, suggesting that GC may lead to accelerated laminin organiztion at the epithelial-mesenchymal interface, leading to epithelial cell differentiation.

Glucocorticosteroids and Sugar Transport

There does appear to be a connection between steroids and sugar transport in a number of tissues. GLUT4 expression in adipose tissue is diminished in response to Dex, while GLUT4 in muscle is increased (Coderre et al., 1996) In adipocytes, Dex increased GLUT4 levels at the plasma membrane in the basal state, while GLUT4 translocation in response to insulin was inhibited.

Oral GC increase intestinal sugar transport. For example, using an in vivo recirculation-perfusion technique in rats, Batt and Peters (1976) showed that 7 days of prednisolone increased intestinal galactose absorption per enterocyte, without influencing the intestinal mucosa or cell kinetics. Similarly, short-term pharmacological doses of prednisolone increased digestive/absorptive function, while paradoxically decreasing the epithelial cell population in rats (Scott et al. 1981). Long-term (28 day) administration of prednisolone or betamethasone in rats increased the activity of BBM proteins and galactose absorption, but induced atrophy of the mucosa and inhibition of cell turnover (Batt and Scott, 1982). Intraluminal administration of hydrocortisone increases glucose absorption (Crake et al., 1984). In this study, the proximal jejunum of humans was perfused with glucose (28 mmol). Intraluminal (100 mg/l) hydrocortisone increased sodium, water and glucose absorption when compared to controls. More recent work in our lab has characterized the effects of steroids on intestinal sugar transport. Thiesen et al. (2003) assessed the influence of the glucocorticosteroids budesonide and prednisone on the in vitro uptake of sugars in weaning male rats. The steroids had no effect on the uptake of D-glucose by SGLT1. In contrast, the uptake of D-fructose by GLUT-5 was increased with both budesonide and prednisone. The increases in the uptake of fructose were not due to variations in the weight of the intestinal mucosa, food intake, or in GLUT-

5 protein abundance or mRNA expression. This enhanced uptake of fructose was likely regulated by posttranslational processes, such as enhancement of the intrinsic activity of the transporters. There were no steroid-associated changes in mRNA expression of c-myc, c-jun, c-fos, proglucagon, or selected cytokines. However, the abundance of ileal ornithine decarboxylase mRNA was increased with Prednisone.

Thiesen et al. (2003) further characterized the effect of steroids using a model of intestinal resection. Adult male Sprague Dawley rats underwent transection or resection of 50% of the middle portion of the small intestine. Prednisone had no effect on the in vitro uptake of glucose or fructose in resected animals. In contrast, in resected rats budesonide increased by over 120% the value of the jejunal maximal transport rate (Vmax) for the uptake of glucose, and increased by over 150% ileal uptake of fructose. Changes in SGLT1, GLUT5, GLUT2, and Na^+K^+-ATPase protein abundance and mRNA expression did not explain the enhancing effect of budesonide. The steroids reduced c-jun, ODC and proglucagon expression. These data suggest that the influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC, and proglucagon, or perhaps other as yet unknown signals.

Early exposure to GC has been associated with lasting effects on cardiovascular, endocrine and metabolic systems. For example, prenatal glucocorticiod exposure results in reduced birth weight (Reinisch et al., 1978; Ikegami et al., 1997; Nyirenda et al., 1998; French et al., 1999; Newnham and Moss, 2001; Bloom et al., 2001), increased blood pressure (Levitt et al., 1996) increased expression of the glucocorticoid receptor in visceral fat (Cleasby et al., 2003), impaired coping in adverse situations (Welberg et al., 2001), increases in corticosterone levels (Levitt et al., 1996; Welberg et al., 2001) hyperglycemia/hyperinsulinemia (Lindsay et al., 1996; Nyirenda et al., 1998; Sugden et al., 2001), increased susceptibility of the inner ear to acoustic noise trauma (Canlon et al., 2003) and increases in PEPCK (rate-limiting enzyme in gluconeogenesis) mRNA and activity (Drake et al., 2005).

Steroids may also influence the development of the retina, although studies have shown both a positive effect on experimentally-induced retinopathy (Rotschild et al., 1999; Yossuck et al., 2001) and an increased risk of retinopathy of prematurity (Haroon et al., 2001). Furthermore, neurodevelopmental problems (Yeh et al., 2004) as well as an increased risk of cerebral palsy (Shinwell et al., 2000) have been documented following postnatal steroid administration.

It is not known if early exposure to GC results in lasting effects on intestinal function. However, because steroids are known to influence intestinal transport in adult animals (Thiesen et al., 2002; Thiesen et al., 2003), and because steroids influence the ontogeny of the intestine (Marti and Fernandez-Otero, 1994; McDonald and Henning, 1992), it seems plausible that they may also play a role in the programming of intestinal function.

GLUCAGON-LIKE PEPTIDE-2 (GLP-2)

Proglucagon is a 160 amino acid peptide encoded by the glucagon gene, and is present in intestinal L cells and α cells of the islets of Langerhans (Bell et al., 1983; Holst, 1994). Proglucagon undergoes post-translational processing in the pancreas, liberating glucagon as the main product. In the intestine, several peptide products (collectively referred to as "enteroglucagon") are produced including glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) (Figure 10).

Both GLP-1 and GLP-2 are secreted from the L cells of the distal small intestine and colon in response to enteral nutrients (Eissele et al., 1992). Both fatty acids (Roberge and Brubaker, 1991) and glucose (Roberge and Brubaker, 1993) stimulate secretion from L cells, but protein meals do not increase GLP-1 or GLP-2 secretion (Elliott et al., 1993; Xiao et al., 1999). However, amino acid mixtures have been shown to stimulate GLP-1 release in humans (Herrmann et al., 1995), and meat hydrolysates do stimulate GLP-1 secretion from rat intestinal L cells *in vitro* (Reimer et al., 2001).

The secretion of these peptides is biphasic, with an early peak within 30 minutes of a meal, followed by a later peak at 60-120 minutes (Morgan et al., 1993; Xiao et al., 1999; Rask et al., 2001). It is thought that neuroendocrine pathways may be responsible for the early secretion, as luminal nutrients are not likely to reach the distal L cells within 30 minutes of ingestion (Ferraris et al., 1990; Roberge and Brubaker, 1991).

The peptides are degraded in the plasma by dipeptidyl peptidase IV (DPPIV) (Tavares et al., 2000) with a half-life for GLP-2 of ~7 minutes (Hartmann et al., 2000). DPPIV-resistant analogs (ALX-600, Teduglutide) with greater potency have been developed for clinical use. These will be discussed in more detail in later sections.

Physiological Effects

While GLP-1 has potent insulinotropic effects (Fehmann and Habener, 1992), GLP-2 has been found to be an important intestinotrophic factor. The relationship between enteroglucagon and small bowel growth was first documented by Gleeson et al. (1971). A patient with an enteroglucagon-producing tumor exhibited small bowel hyperplasia, and injection of the tumor extract into mice resulted in intestinal growth (Bloom and Polak, 1982). Drucker et al. (1996) demonstrated that GLP-2 was the specific agent responsible for this effect. GLP-2 appeared to exert a "tissue specific" effect on the gut, as no changes were found in other tissues including spleen, heart, brain and liver.

GLP-2 given subcutaneously to adult mice (6-43 µg, twice daily, for 10 days) increases small bowel weight and crypt-villous height (Drucker et al., 1997; Tsai et al., 1997). Intestinal proliferation was increased, and apoptosis was reduced. The effect of GLP-2 was not due to changes in food consumption. The effects of GLP-2 were sustained, as increases in growth were still evident after three months of administration. The rapid turnover of the epithelium was thought to contribute to the lack of densensitization that was observed with prolonged GLP-2 treatment.

In addition to its morphological effects, GLP-2 (when administered intravenously or subcutaneously) also increases the activity and expression of BBM enzymes including SI, LPH, maltase-glucoamylase and aminopeptidase N (Petersen et al., 2002; Brubaker et al., 1997; Kitchen et al., 2000). Although the effects on gastric physiology have been conflicting (Schmidt et al., 2003; Nagell et al., 2004), inhibition of gastric emptying in pigs has been observed with GLP-2 treatment (Wodjemann et al., 1998). Reductions in meal-stimulated gastric acid secretion have also been observed with GLP-2 administration in humans (Wojdemann et al., 1999). GLP-2 enhances barrier function in murine intestinal epithelium (Benjamin et al., 2000), making it a potential therapeutic for disorders such as inflammatory bowel disease and necrotizing pancreatitis, both of which are characterized by increases in intestinal permeability (Benjamin et al., 2000; Kouris et al., 2001).

Intestinal Resection

Plasma GLP-2 levels rise following intestinal resection in rats (Topstad et al., 2001; Thulesen et al., 2001; Ljungmann et al., 2001). When rats were subjected to a 70% midjejunoileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP-2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats (Dahly et al., 2003). While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 days post-resection. These data support a significant role for endogenous GLP-2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats.

GLP-2 administration in rats increases the adaptive response to massive intestinal resection (Scott et al., 1998). Sprague-Dawley rats were divided into two groups, with a 75% mid-jejunum-ileum resection and a sham operated group. Animals were given 0.1 μg/g GLP-2 analog (protease resistant human GLP-2) or placebo given subcutaneously twice daily for 21 days. Administration of the GLP-2 analog was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum.

Martin et al. (2004) investigated the effects of GLP-2 in a total parenteral nutrition (TPN)-supported model of experimental short bowel syndrome. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection, and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, or TPN + 10 μg/kg/h GLP-2. TPN plus GLP-2 treatment resulted in increased bowel and body weight, villous height, intestinal mucosal surface area, crypt cell proliferation, and reduced intestinal permeability, as compared with the TPN alone animals. GLP-2 increased serum GLP-2 levels and intestinal SGLT-1 protein abundance as compared with either TPN or enteral groups. This demonstrates that GLP-2 is capable of stimulating intestinal adaptation in the absence of enteral feeding,

Because a number of hormones and growth factors have been shown to influence intestinal function, Washizawa et al. (2004) compared the effects of GLP-2, growth hormone (GH) and keratinocye growth factor (KGF) on markers of gut adaptation following massive small bowel resection (MSBR). KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in both the small bowel and the colon. While both GH and KGF increased colonic mucosal growth, GLP-2 exerted superior trophic effects on jejunal growth. GLP-2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP-2, GH and KGF on gut adaptation following MSBR, the authors conclude that a combination of these agents may be most beneficial.

Human studies with GLP-2 have also been performed. A non-placebo controlled study was conducted in 8 patients with SBS with an end-enterostomy type of anastomasis (6 had Crohn's disease and 4 were not receiving HPN) (Jeppesen et al., 2001). Treatment with GLP-2 (400 μg subcutaneously twice a day for 35 days) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in 5 and 6 patients, respectively.

Jeppesen (2003) concludes that *"Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research studies. The optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of parenteral nutrition in a larger fraction of short-bowel patients".*

While the mechanism by which GLP-2 stimulates the adaptive response is unknown, its effects on an immediate early gene, PC4/TIS7, have been observed. In postconfluent, quiescent IEC 18 cells in culture, a stable derivative of GLP-2, r(gly2)GLP-2, increased PC4/TIS7 expression. r(gly2)GLP-2 administered intraperitoneally to mice similarly induced increased PC4/TIS7 mRNA compared with vehicle control (Swietlicki et al., 2003).

A number of studies aimed at establishing the effect of GLP-2 on intestinal transport. Cheeseman and Tsang (1996) showed that a two hour vascular perfusion of GLP-2 (4 hr, 400 and 800 pM) in rats increased D-glucose Vmax by approximately 65%in the basolateral membrane, and by three-fold in the BBM, with a concomitant increase in SGLT1 protein (Cheeseman, 1997). An anti-GLP antibody abolished the GLP-2-induced increase in transport (Cheeseman and Tsang, 1996).

Not all studies have demonstrated an effect of GLP-2 on nutrient transport. For example, Brubaker et al. (1997) treated mice subcutaneously with GLP-2 (2.5 μg) for a 10 day period, and failed to reveal increases in the absorption of glucose or maltose. Curiously, an increased capacity for nutrient digestion was found to be due to increases in BBM enzyme activities, resulting in an apparent uncoupling of digestive capacity from subsequent sugar absorption. It is unclear why there is this discrepancy in the results of these studies on the effect of GLP-2 on nutrient uptake.

The rapid trafficking of the glucose and fructose transporter GLUT2 into the BBM following a glucose infusion or meal contributes to sugar uptake (Helliwell et al., 2000a; Helliwell et al., 2000b; Kellett and Helliwell, 2000) (please see section 1.1a). GLUT2 protein levels in the BBM increased two-fold when luminal perfusions were increased from 0 mM to 100 mM glucose. A onehr vascular infusion of GLP-2 doubled the rate of fructose absorption following luminal fructose perfusion (Au et al., 2002). Fructose absorption in this study was determined by the appearance of fructose in the vascular bed. Western blotting of biotinylated surface-exposed protein showed a doubling of GLUT2 expression in the BBM following GLP-2 infusion. Thus, GLP-2 may promote the insertion of GLUT2 into the BBM, thereby providing a low affinity/ high capacity route in addition to SGLT1 and GLUT5 by which fructose or glucose may be absorbed into the enterocyte.

Ramsanahie et al. (2004) examined the effect of chronically administered GLP-2 on diurnal SGLT1 expression. Rats were treated with [Gly2]GLP-2 (twice daily; 1microg/g body weight) or vehicle (control) for 10 days. GLP-2 administration did not alter the diurnal increase in mRNA levels of SGLT1, GLUT2, or GLUT5.

However, SGLT1 protein was increased three-fold by GLP-2, and *in situ* hybridization showed that SGLT1 mRNA was distributed along the entire length of the villi. This is in contrast to what was seen in control animals, where SGLT1 was restricted to the mid and upper villus, with less SGLT1 mRNA localized to the villous tip. Also, in contrast to the diffuse staining seen in control animals, immunofluorescence microscopy showed that SGLT1 protein in GLP-2 treated animals was preferentially localized to the BBM, with little or no staining in the cytoplasm. This may represent another mechanism by which GLP-2 increases intestinal glucose uptake.

The effect of GLP-2 on *in vivo* nutrient absorption may also be attributed to nitric oxide-dependent increases in intestinal blood flow. Infusing GLP-2 (500 pmol/kg/h) for 4 hours in TPN-fed piglets led to increased portal-drained visceral (PDV) blood flow rate, intestinal blood volume, and PDV glucose uptake. GLP-2 also increased intestinal constitutive nitric oxide synthase (NOS) activity and endothelial NOS protein abundance (Guan et al., 2003). Thus, in TPN-fed neonatal pigs, GLP-2 acutely stimulates intestinal blood flow and glucose utilization, and that this response is nitric oxide-dependent.

Intestinal Development

A role for GLP-2 in the ontogeny of the intestine has been proposed. Lee et al. (1990) established that proglucagon mRNA was detectable in the rat fetus, and that immunoreactivity increased in the early neonatal period. Prohormone convertases, which are required for the liberation of GLP-2 from proglucagon, are also detected in the fetal rat. Lovshin et al. (2000) detected GLP-2R mRNA during fetal and neonatal development in the rat, with levels being higher in the fetal and neonatal gut as compared to adult rats. High levels of GLP-2 (1-33) were also detected in the circulation of 13 day old neonatal rats, and GLP-2 immunoreactivity was found in the fetal rat intestine. In order to prove that fetal cells were capable of secreting GLP-2, fetal rat intestinal cell cultures were studied, and were found to secrete correctly processed GLP-2 (1-33). The administration of a degradation resistant GLP-2 analog (h[Gly2]-GLP-2) to 1 day old rat pups for a period of 10 days resulted in increases in both small bowel weight and length. Thus, the GLP-2/GLP-2R axis is functional in early life, and that the developing intestine is capable of synthesizing, secreting and responding to GLP-2.

A subsequent study done by Petersen et al. (2002) on premature (92% gestation) TPN-fed piglets demonstrated that in addition to increases in maltase mRNA and activity, increases in SI and aminopeptidase N activities were observed. Thus, it appears that some of the effects that GLP-2 exerts on intestinal function may be related to gestational age at birth, as the premature intestine was more responsive to exogenous GLP-2 than the term neonatal intestine. The authors also noted that GLP-2 infused into pig fetuses *in vivo* passed into the maternal circulation (Petersen et al., 2001). This suggests that GLP-2 may pass through the placenta, and conversely, may expose the fetus to maternal GLP-2. This raises the possibility that GLP-2 given to pregnant animals may alter the form or function of the offspring.

The actions of GLP-2 are transduced by the GLP-2 receptor (GLP-2R), which was characterized and cloned by Munroe et al. (1999). GLP-2R was detected in several rat tissues including the stomach, small bowel, colon, and in small quantities in other tissues such as brain and lung (Munroe et al., 1999). This raises the possibility that GLP-2 may have effects beyond the small intestine.

The receptor is a G-protein coupled receptor with 7 transmembrane domains, and is encoded by a single gene localized to chromosome 17p13.3. GLP-2R expression is highest in the proximal small intestine, and decreases distally along the longitudinal axis (Munroe et al., 1999). GLP-2R has been localized to intestinal enteroendocrine cells in humans using immunohistochemistry (Yusta et al., 2000a), to enteric neurons in mice using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (Bjerknes and Cheng, 2001), and to rat enterocytes using ^{125}I-GLP-2 (Thulesen et al., 2000). Orskov et al. (2005) found GLP-2 receptors mainly on subepithelial myofibroblasts in rat, mouse, and human small and large intestine by immunohistochemistry and *in situ* hybridization. By double labelling they found that these GLP-2 receptor immunoreactive cells also produce smooth muscle actin and keratinocyte growth factor (KGF). KGF antibody abolished the growth promoting effect of GLP-2 in the large intestine, but not in the small intestine. This suggests that GLP-2 in the gut may act by activating receptors on the subepithelial myofibroblasts, thereby causing the release of growth factors, which in turn stimulate intestinal growth. Therefore, at this time it remains unclear whether the intestinotropic effects of GLP-2 are due to direct effects on enterocytes or are mediated by secondary factors.

Even before the GLP-2 receptor was cloned, the role of the PI3K pathway in mediating the effects of GLP2 on intestinal sugar transport was studied by Cheeseman et al. (1997).*In vivo* infusions of GLP-2 produced an acceleration of sodium-dependent glucose uptake into BBM vesicles, with similar increase in SGLT-1 abundance. The effect of GLP-2 could be inhibited by luminal brefeldin A (which blocks protein trafficking from the Golgi to the plasma membrane), or by the PI3K inhibitor, wortmannin. These results indicate that GLP-2 is able to induce trafficking of SGLT-1 from an intracellular pool into the BBM and that PI3K may be involved in the intracellular signaling pathway in this response.

Because the GLP-2R is not expressed on any intestinal cell lines, *in vitro* studies on GLP-2 receptor signalling have been carried out in transfected heterologous cell types. Work done on transfected baby hamster kidney fibroblasts showed that GLP-2 stimulated AP-1 dependent pathways increased cAMP, but did not change intracellular calcium levels (Yusta et al., 1999). There is decreased apoptosis and reduced caspase-3 activation following GLP-2 treatment *in vitro* (Yusta et al., 2000). PKA, PI3K and the ERK pathways were not found to be essential for GLP-2 inhibition of apoptosis, which was associated with reductions in cytochrome c release and cleavage of poly ADP-ribose polymerase (PARP).

GLP-2-treated Caco2 cells (10 μm, 3 days) demonstrated a greater than 10-fold increase in proliferation (Jasleen et al., 2000). This response was inhibited by PI3K and mitogen activated/extracellular signal-regulated kinase (MEK) inhibitors. A significantly greater abundance of the phosphorylated forms of both ERK-1 and ERK-2 was present in cells following GLP-2. This suggests that the increase in Caco-2 proliferation in response to GLP-2 may be due, at least in part, to the involvement of both the PI 3-kinase and the MAPK pathways.

The limitations of this work, however, centers around the fact that both the authors of this study as well as Yusta et al. (2000) were unable to show, using

Western blotting or RT-PCR, that Caco2 cells express endogenous GLP-2R. This suggests that the proliferative effect of GLP-2 on Caco2 cells may be mediated by other receptors, such as the EGFR, which mediates GLP-1 induced proliferation in pancreatic β cells (Buteau et al. (2003). Furthermore, the observation that GLP-2 is able to induce proliferation in a tumor cell lines, raises a concern regarding the role of GLP-2 in promoting tumor growth. Indeed, recent work by Thulesen et al. (2004) showed that GLP-2 promotes the growth of mucosal neoplasms in female C57bl mice whose colonic tumours were experimentally induced by administering a methylating carcinogen.

The intestinotropic effects of GLP-2 have resulted in it being considered as a candidate for clinical use in patients with compromised intestinal function. Using an animal model of parenterally fed premature pigs, Burrin et al. (2000) examined whether exogenous glucagon-like peptide (GLP)-2 infusions stimulate intestinal growth. Piglets (106-108 days gestation) were given TPN, or TPN + human GLP-2 (25 nmol/kg/d). Crypt cell proliferation and apoptosis were measured *in situ* by 5-bromodeoxyuridine (BrdU) and terminal dUTP nick-end labeling (TUNEL), respectively. Intestinal protein and DNA accretion rates and villus heights were increased with GLP-2, while GLP-2 decreased fractional protein degradation rate. Apoptosis, as measured by the percentage of TUNEL-positive cells, was reduced with GLP-2, while no changes were seen in the percentage of proliferating BrdU-positive crypt cells. Thus, GLP-2 increases intestinal growth in premature, TPN-fed pigs by decreasing proteolysis and apoptosis, and enteral nutrition was not required for these effects to occur.

Proliferation and Apoptosis

Yusta et al. (2002) demonstrated that GLP-2, in a cycloheximide-insensitive manner, enhanced survival in baby hamster kidney cells stably transfected with the rat GLP-2R; reduced mitochondrial cytochrome c efflux, and attenuated the caspase-dependent cleavage of Akt, poly(ADP-ribose) polymerase, and beta-catenin following inhibition of phosphatidylinositol 3-kinase (PI3K) by LY294002. The prosurvival effects of GLP-2 on LY294002-induced cell death were independent of Akt, p90(Rsk), or p70 S6 kinase activation; were mimicked by forskolin; and were abrogated by inhibition of protein kinase A (PKA) activity. GLP-2 inhibited activation of glycogen synthase kinase-3 (GSK-3) through phosphorylation at Ser(21) in GSK-3alpha and at Ser(9) in GSK-3beta in a PI3K-independent, PKA-dependent manner. GLP-2 reduced LY294002-induced mitochondrial association of endogenous Bad and Bax and stimulated phosphorylation of a transfected Bad fusion protein at Ser(155) in a PI3K-independent, but H89-sensitive manner, a modification known to suppress Bad pro-apoptotic activity. These results suggest that GLP-2R signaling enhances cell survival independently of PI3K/Akt by inhibiting the activity of a subset of pro-apoptotic downstream targets of Akt in a PKA-dependent manner.

Rocha et al. (2004) assessed the proliferative actions of GLP-2 on the human Caco-2 cell line. GLP-2 stimulated proliferation was inhibited in a dose-dependent fashion by both pertussis and cholera toxin (specific G protein inhibitors). This suggests that a G-protein-linked signaling pathway is involved with GLP-2 bioactivity in Caco-2 cells. GLP-2 stimulated proliferation was also augmented by 2',5'-dideoxyadenosine, which increases adenylate cyclase. Proliferation rates were inversely proportional to changes in intracellular cAMP concentration. These findings suggest that a G-protein linked signaling pathway is involved with GLP-2 bioactivity in the intestinal epithelial cell line Caco-2.

Walsh et al. (2003) studied GLP-2 signalling in isolated rat intestinal mucosal cells expressed mRNA transcripts for the GLP-2R, as well as for chromogranin A and beta-tubulin III, markers for enteroendocrine and neural cells, respectively. cAMP production in response to [Gly2]GLP-2, a degradation-resistant analog of GLP-2, was maximal at 10^{-11} M with reduced cAMP accumulation observed at higher doses. The cAMP response was abolished by pretreatment with 10^{-6} M GLP-2, indicating receptor desensitization. GLP-2 treatment of isolated mucosal cells increased ^{3}H-thymidine incorporation, and this was prevented by inhibition of the protein kinase A pathway. In contrast, GLP-2 did not affect p44/p42 MAPK phosphorylation or the levels of cytosolic calcium in the mucosal cell preparation. These results provide evidence that activation of the endogenous rat mucosal GLP-2 receptor is linked to activation of a cAMP/protein kinase A-dependent, growth-promoting pathway *in vitro*.

Estall et al. (2004) examined the mechanisms regulating signaling, internalization, and trafficking of the GLP-2R to identify determinants of receptor activation and desensitization. Heterologous cells expressing the transfected rat or human GLP-2R exhibited a rapid, dose-dependent, and prolonged desensitization of the GLP-2-stimulated cAMP response and a sustained GLP-2-induced decrease in levels of cell surface receptor. Surprisingly, inhibitors of clathrin-dependent endocytosis failed to significantly decrease GLP-2R internalization, whereas cholesterol sequestration inhibited ligand-induced receptor internalization and potentiated homologous desensitization. The hGLP-2R localized to both Triton X-100-soluble and -insoluble (lipid raft) cellular fractions and colocalized transiently with the lipid raft marker caveolin-1. Although GLP-2R endocytosis was dependent on lipid raft integrity, the receptor transiently associated with green fluorescent protein tagged-early endosome antigen 1-positive vesicles and inhibitors of endosomal acidification attenuated the reappearance of the GLP-2R on the cell surface. This data demonstrates that GLP-2R desensitization and raft-dependent trafficking represent distinct and independent cellular mechanisms and provide new evidence implicating the importance of a clathrin- and dynamin-independent, lipid raft-dependent pathway for homologous G protein-coupled receptor internalization.

Clinical

Burrin et al. (2005) studied 38 TPN-fed neonatal piglets infused intravenously with either saline or GLP-2 at three rates (2.5, 5.0, and 10.0 nmol/kg/d for 7 d). GLP-2 infusion dose-dependently increased small intestinal weight, DNA and protein content, and villus height; however, stomach protein synthesis was decreased by GLP-2. Intestinal crypt and villus apoptosis decreased and crypt cell number increased linearly with GLP-2 infusion rates, whereas cell proliferation and protein synthesis were stimulated only at the high GLP-2 dose. The intestinal activities of caspase-3 and -6 and active caspase-3 abundance decreased, yet procaspase-3 abundance increased markedly with increasing infusion rate and plasma concentration of GLP-2. The GLP-2-dose-dependent suppression of intestinal apoptosis and caspase-3 activity was associated with increased protein kinase B and glycogen-synthase kinase-3 phosphorylation, yet the expression phosphatidylinositol 3-kinase was unaffected by GLP-2. Intestinal endothelial nitric oxide synthase mRNA and protein expression was increased, but only at the high GLP-2 dose. These authors concluded that the stimulation of intestinal epithelial survival is concentration-dependent at physiological GLP-2 concentrations.However, induction of cell proliferation and protein synthesis is a pharmacological response. Moreover, they showed that GLP-2 stimulates intestinal cell survival and proliferation in association with induction of protein kinase B and glycogen-synthase kinase-3 phosphorylation and Bcl-2 expression.

Koehler et al. (2005) identified several expressed sequence tags from human cervical carcinoma cDNA libraries that correspond to GLP-2R nucleotide sequences. GLP-2R mRNA transcripts were detected by RT-PCR in HeLa cervical carcinoma cells and Ca Ski cervical carcinoma cells. GLP-2 increased cAMP accumulation and activated ERK1/2 in HeLa cells transiently expressing the cloned human HeLa cell GLP-2R cDNA. However, the GLP-2R-induced activation of ERK1/2 was not mediated through Gαs, adenylyl cyclase, or transactivation of the epidermal growth factor receptor, but was pertussis toxin sensitive, inhibited by dominant negative Ras, and dependent on betagamma-subunits. GLP-2 also induced a significant increase in bromodeoxyuridine incorporation that was blocked by dominant negative Ras. Furthermore, GLP-2 inhibited HeLa cell apoptosis induced by LY294002 in a protein kinase A-dependent, but ERK-independent, manner. These findings demonstrate that the HeLa cell GLP-2R differentially signals through both G(alpha)s/cAMP- and G(i)/G(o)-dependent pathways, illustrating for the first time that the GLP-2R is capable of coupling to multiple heterotrimeric G proteins defining distinct GLP-2R-dependent biological actions.

Finally, the effects of GLP-2 may be due to transactivation of other cell surface receptors. For example, GLP-1 increases PI3K activity and enhances beta-cell proliferation via transactivation of the EGFR (Buteau et al., 2003).

Indeed, Haderslev et al. (2002) found that GLP-2 administration significantly increased spinal bone mineral density in short-bowel patients with no colon. Henrikson et al. (2004) also found beneficial effects of GLP-2 on bone resorption. Centrally administered GLP-2 increases satiety in rodents (Tang-Christensen et al., 2000; Lovshin et al. 2001), while peripherally administered GLP-2 does not influence gastric emptying, food intake or satiety in humans (Schmidt et al., 2003; Sorensen et al., 2003).

Although the effects of early exposure to hormones such as GLP-2 are unknown, the presence of circulating GLP-2 and the detection of the GLP-2R in fetal and neonatal rats (Lovshin et al., 2000) suggests a role for GLP-2 in regulating intestinal development. Drozdowski et al., unpublished observations, 2005) has shown that GLP-2 modifies intestinal morphology when given to suckling rats, and enhances sugar uptake into the intestine of rats whose mothers were given GLP-2 during pregnancy and lactation.

Therefore, it is possible that exposing young animals to GLP-2, either directly or via their pregnant and lactating dams, may also have lasting effects on intestinal function.

GLP-2 and GLUCOCORTICOSTEROIDS

The possibility of a growth factor or hormone acting additively or synergistically with a steroid hormone has been demonstrated in various tissues. For example, EGF potentiates the proliferative effects of progesterone and estrogen in the mammary gland (Haslam et al., 1993). In a review, Lange (2004) discusses the cross-talk that occurs between steroid hormone receptors and intracellular signalling pathways. There is clearly evidence of non-genomic and extra-nuclear functions of steroid receptors, including the initiation of signal transduction pathways. Indeed, this type of cross-talk may explain how genes are co-ordinately regulated by mitogenic stimuli in hormone responsive tissues. For example, Migliaccio et al. (1996) reported MAPK activation by estradiol, and interactions between the progesterone receptor, the estrogen receptor and p60-Src kinase. A synergistic effect between Dex and GLP-2 may occur in the intestine, as GC have a permissive effect on several hormones including catecholamines, thyroid hormones, growth hormone and ACTH (Baxter et al., 1976). GC mediates a permissive action, mostly for hormones which act on G-protein coupled receptors, and increases adenylate cyclase (Michel et al. 1994; Meier, 1997). This occurs because Dex alters adenylyl cyclase, enhancing the effects of cAMP generating agonists. Therefore, an interaction between DEX and GLP-2 is likely, as the GLP-2 receptor is a G-protein coupled receptor.

Furthermore, cAMP and PKA, which are activated by GPCRs like GLP-2R, may increase the steroid sensitivity of a target cell by increasing the DNA binding ability of the GR for its response elements. In a study by Rangarajan et al. (1992) using embryonal carcinoma cells lacking cAMP response element binding protein (CREB), activation of PKA increased hormone-dependent trans-activation of the GR. The effect of PKA was related to the DNA binding domain of the GR, as deletion of the amino-terminal or the ligand-binding domain did not alter PKA's effect. However, the absence of a consensus PKA phosphorylation site within the GR DNA binding domain led the authors to suggest that the GR is not a direct substrate for phosphorylation by PKA. Instead, they propose a multi-step process involving other cellular kinases and phosphatases that may interact with the GR.

As discussed previously, there are several pieces of evidence that suggest that GLP-2 may exert its effect via the PI3K pathway. Steroid receptors may interact with this pathway as Simoncini et al. (2000) showed that the estrogen receptor binds to p85 subunit of PI3K.

Although there are no reports of the effect of Dex on PI3K in the intestine, an association has been observed in other tissues. For example, Saad et al. (1994) showed that Dex induced a 69% increase in the level of PI 3-kinase in adipocytes as determined by immunoblotting. Conversely, Buren et al. (2002) demonstrated that Dex decreases PI3K, PKB, insulin-stimulated PKB phosphorylation and glucose transport in isolated rat adipocytes, without changes in GLUT4. These results suggest that glucocorticoids, independently of the surrounding glucose and insulin concentration, impair glucose transport capacity in fat cells. Finally, Krasil'nikov et al. (1999) showed that prolonged exposure to Dex increased PI3K in Rous sarcomavirus-transformed hamster fibroblasts.

Certainly in other tissues, such as adipose and muscle, PI3K is involved in regulating insulin-stimulated glucose uptake (reviewed Furtado et al., 2002). In the intestine, EGF stimulated increases in intestinal glucose transport in rabbits is abolished by the PI3K inhibitor LY294002 (Millar et al., 2002). Similarly, IGF-1 associated increases in jejunal glucose uptake and Na^+K^+-ATPase activity are abolished by wortmannin, another PI3K inhibitor (Alexander and Carey, 2001). So clearly this pathway plays an important role in transducing signals from hormones and growth factors to the proteins involved in sugar transport.

2.6. FIGURES AND TABLES

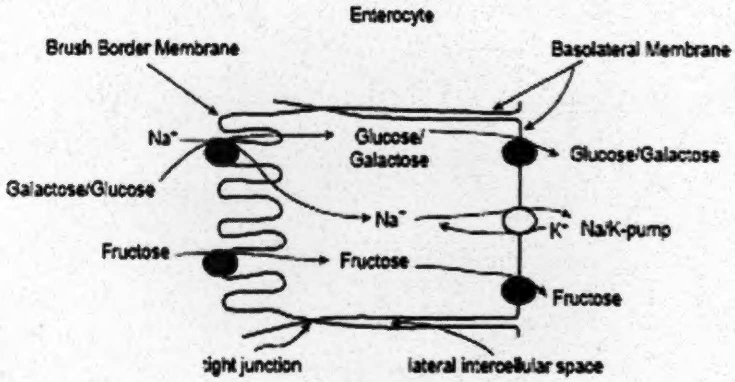

Enterocyte
Brush Border Membrane
Basolateral Membrane
Na⁺
Glucose/
Galactose
Glucose/Galactose
Galactose/Glucose
Na⁺
K⁺
Na/K-pump
Fructose
Fructose
Fructose
tight junction
lateral intercellular space

Figure 1. Classical model of intestinal sugar transport (from Wright et al., 1998)

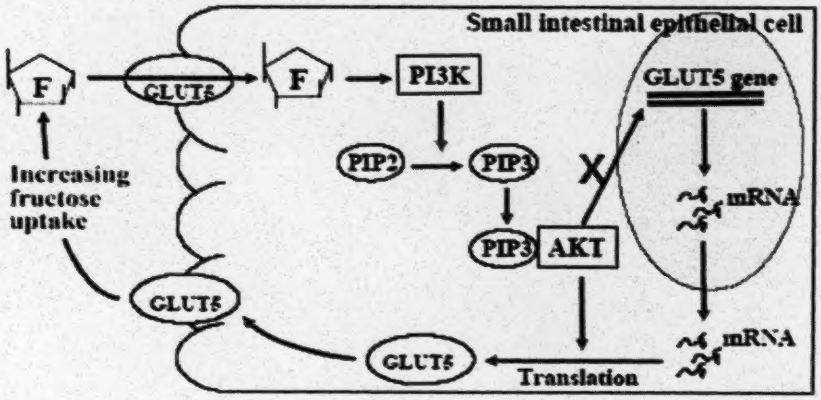

Small intestinal epithelial cell
F
GLUT5
F
PI3K
GLUT5 gene
PIP2
PIP3
X
mRNA
Increasing
fructose
uptake
PIP3
AKT
GLUT5
mRNA
GLUT5
Translation

Figure 2. Proposed role of PI3K/Akt signalling pathway in the regulation of GLUT5 synthesis and trafficking (from Cui et al., 2005)

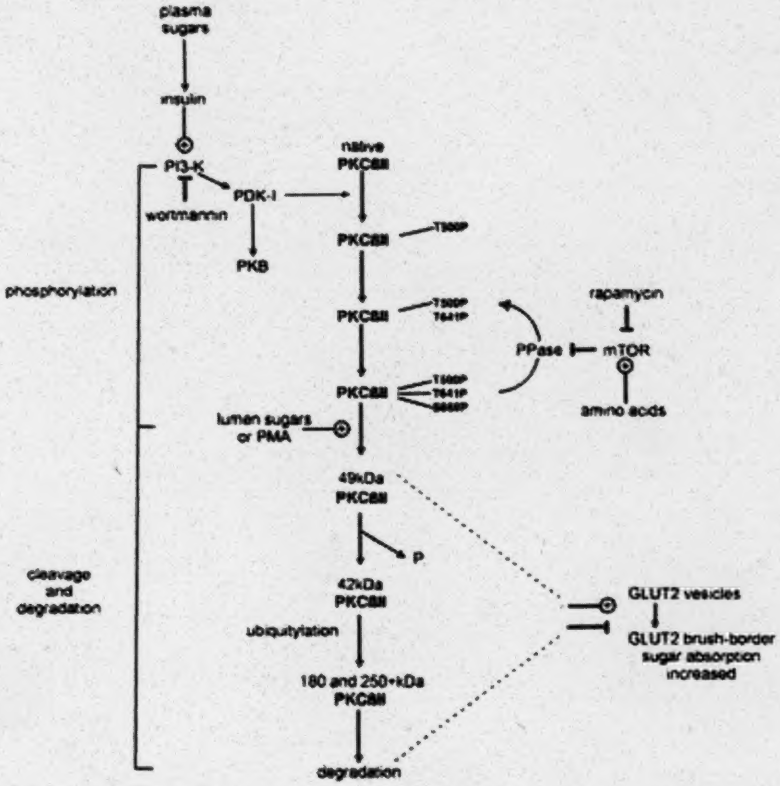

plasma sugars
insulin
PI3-K
wortmannin
PDK-I
PKB
native PKCβII
PKCβII
T500P
PKCβII
T500P
T641P
PKCβII
T500P
T641P
S660P
phosphorylation
rapamycin
PPase
mTOR
amino acids
lumen sugars or PMA
49kDa PKCβII
P
42kDa PKCβII
cleavage and degradation
ubiquitylation
180 and 250+kDa PKCβII
degradation
GLUT2 vesicles
GLUT2 brush-border sugar absorption increased

Figure 3. Potential signaling pathways for the regulation of GLUT2-mediated sugar absorption by insulin and amino acids through the control of PKC βII activity. (from Helliwell et al., 2003).

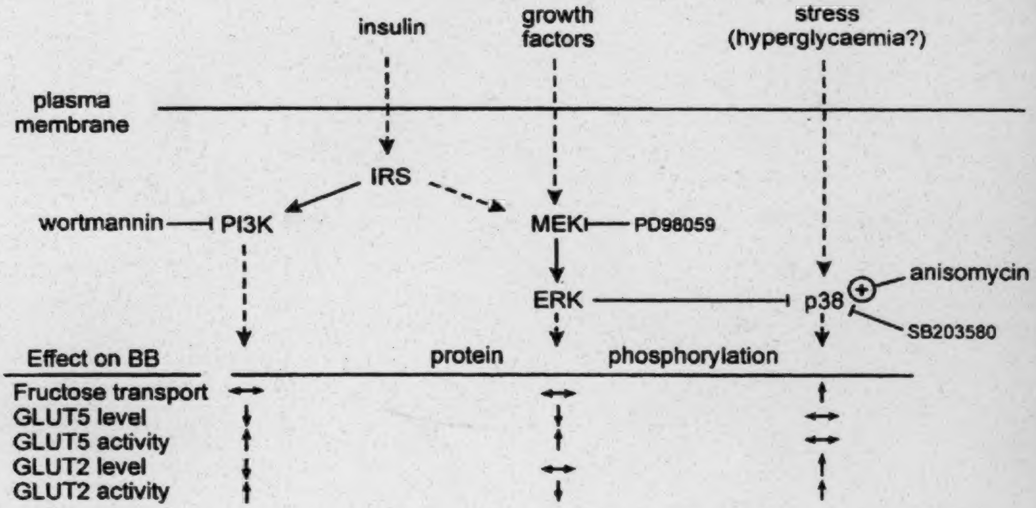

insulin
growth factors
stress (hyperglycaemia?)
plasma membrane
IRS
wortmannin
PI3K
MEK
PD98059
ERK
p38
anisomycin
SB203580
Effect on BB
protein phosphorylation
Fructose transport
GLUT5 level
GLUT5 activity
GLUT2 level
GLUT2 activity

Figure 4. The regulation of BBM fructose transport by the PI3K, ERK and p38 MAPK signalling pathways (from Helliwell et al., 2000a).

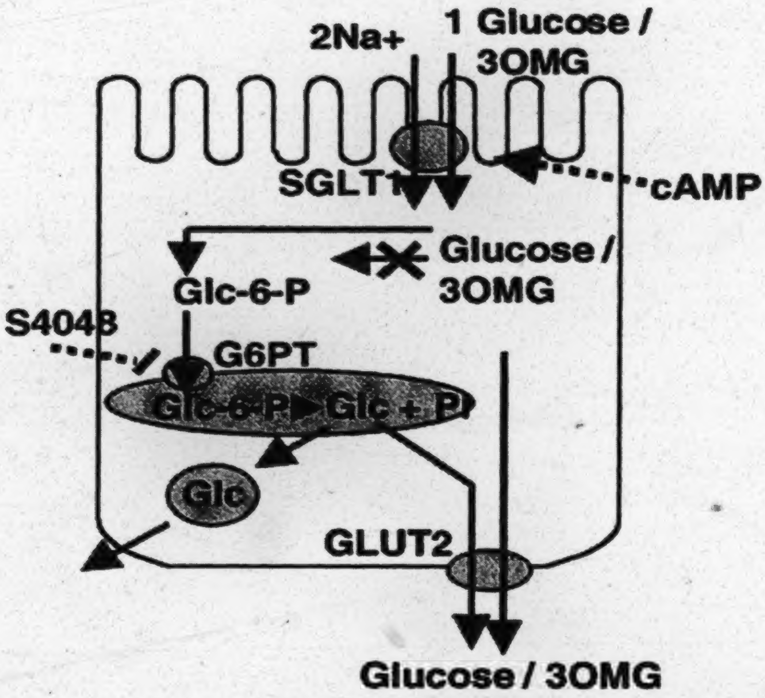
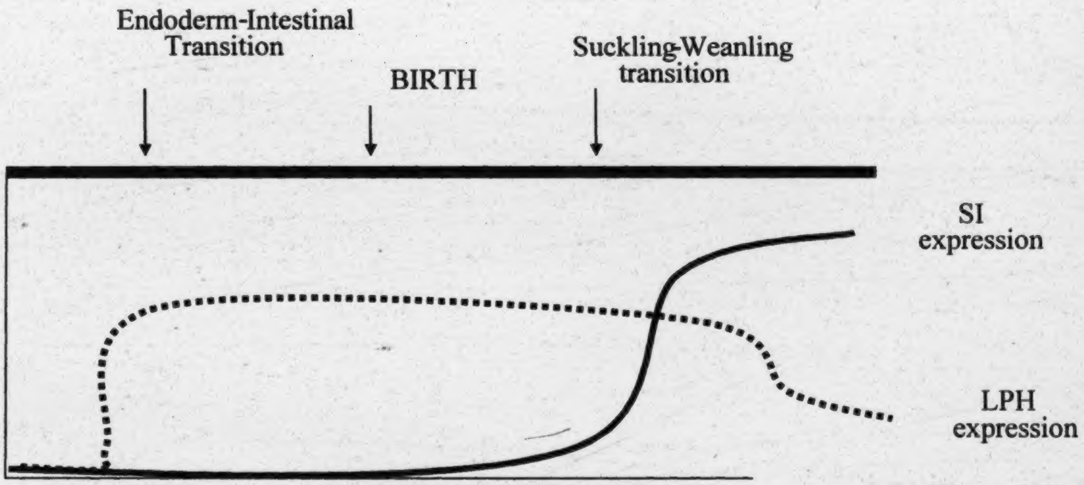
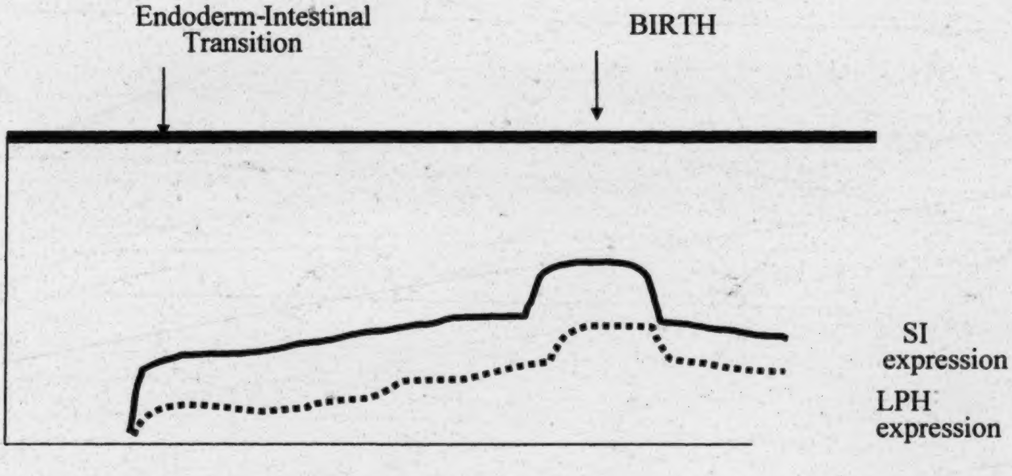

2Na+
1 Glucose / 3OMG
SGLT1
cAMP
Glucose / 3OMG
Glc-6-P
S4048
G6PT
Glc-6-P
Glc + Pi
Glc
GLUT2
Glucose / 3OMG

Figure 5. Proposed alternative mechanism for intestinal glucose transepithelial transport (from Stumpel et al., 2001).



RODENT



HUMAN

Figure 6. Regulation of SI and LPH gene expression during development of the rodent and the human small intestine. Adapted from Traber (2000) in *Development of the Gastrointestinal Tract.* Eds Sanderson IR and Walker WA. BC Decker Inc, Hamilton.

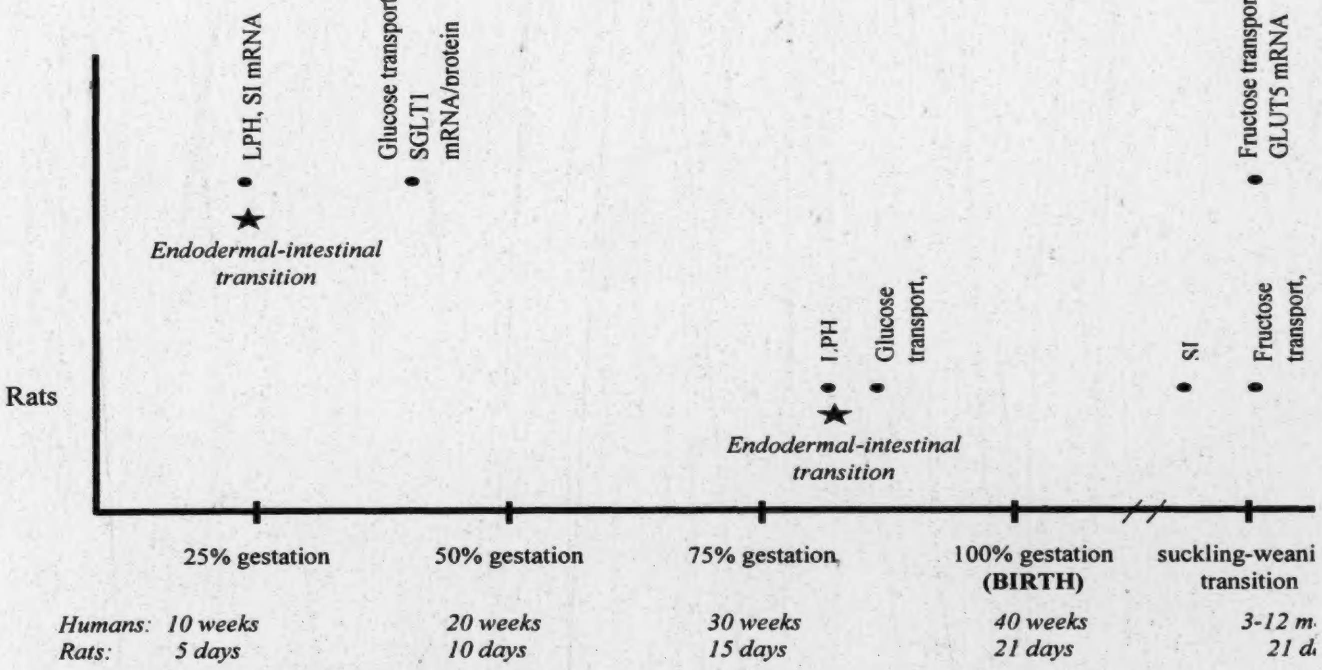

LPH, SI mRNA
Glucose transport, SGLT1 mRNA/protein
Fructose transport, GLUT5 mRNA
Endodermal-intestinal transition
Rats
LPH
Glucose transport,
SI
Fructose transport,
Endodermal-intestinal transition
25% gestation
50% gestation
75% gestation
100% gestation (BIRTH)
suckling-weani transition
Humans: 10 weeks
Rats: 5 days
20 weeks
10 days
30 weeks
15 days
40 weeks
21 days
3-12 m
21 d

Figure 7. The prenatal and postnatal appearance of BBM disaccharidases and intestinal sugar transporters in rats and human.

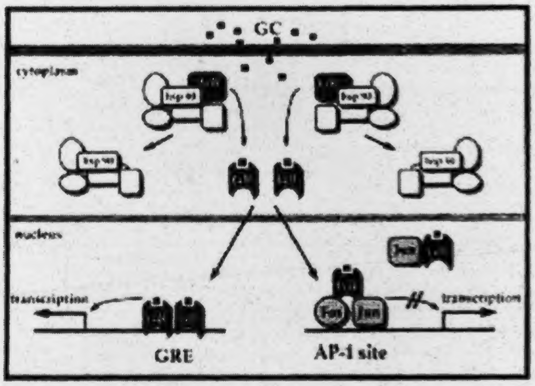
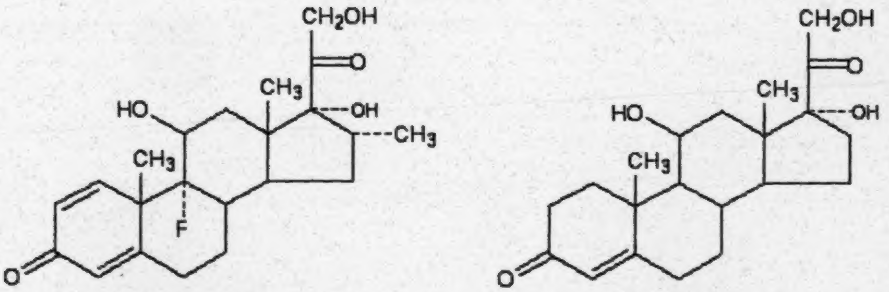

GC
cytoplasm
hsp 90
nucleus
Jun
transcription
Fos
Jun
transcription
GRE
AP-1 site

Figure 8. Simplified model of GR-mediated transcriptional modulation (from Bamberger et al. 1996)


CH2OH
CH3
HO
OH
CH3
CH3
F
O
CH2OH
CH3
HO
OH
CH3
O

Dexamethasone
MW 392.5

Cortisol
MW 362.5

Figure 9. The structure of synthetic and naturally occurring glucocorticosteroids.

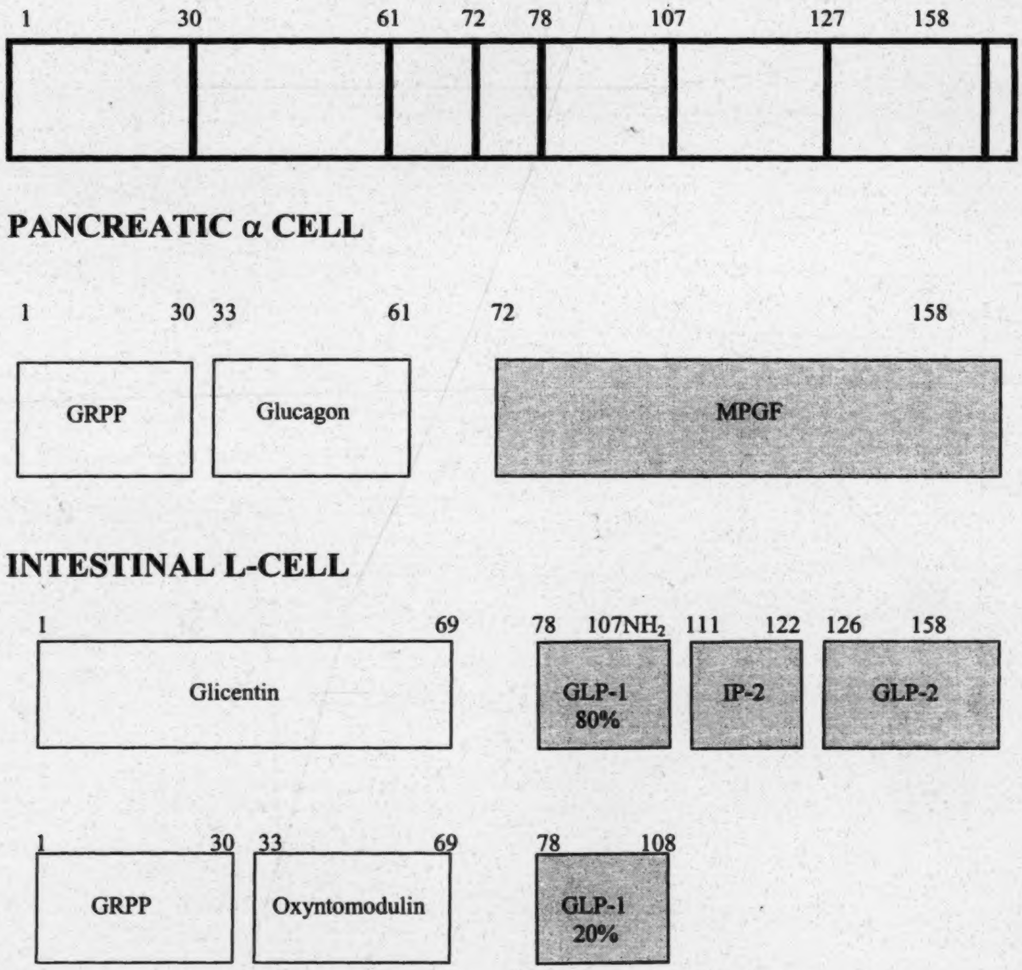

1
30
61
72
78
107
127
158
PANCREATIC α CELL
1
30
33
61
72
158
GRPP
Glucagon
MPGF
INTESTINAL L-CELL
1
69
78
107NH₂
111
122
126
158
Glicentin
GLP-1 80%
IP-2
GLP-2
1
30
33
69
78
108
GRPP
Oxyntomodulin
GLP-1 20%

Figure 10. Post-translational processing of proglucagon in the pancreas and the intestinal L-cells. The numbers represent the amino acid at which enzymatic cleavage occurs.

Transporter	Km
SGLT1 (BBM)	Glucose:6 mM Fructose:>100 mM *(Wright et al., 2003)*
GLUT2 (BLM)	Glucose:> 50 mM Fructose: 66 mM *(Walmsley et al., 1998)*
GLUT5 (BBM)	Fructose: 6-14 mM *(Walmsley et al., 1998)*

Table 1. Affinity constants of the sugar transporters

	INHIBITORS
SGLT1	Phloridzin
GLUT2	Cytochalasin B Phloretin
GLUT5	Glyco-1,3-oxazolidin-2-thiones, -ones *(Girniene et al., 2003)*
Na^+K^+-ATPase	Oubain

Table 2. Inhibitors of sugar transporters

Factors influencing SGLT1 function	Factors influencing GLUT2 function	Factors influencing GLUT5 function
Foxl1 *(Katz et al., 2004)*	PKCβII*(Helliwell et al., 2000b)*	cAMP *(Mahraoui et al., 1994)*
AMPK *(Walker et al., 2004)*	p38*(Helliwell et al., 2000a)*	p38 *(Helliwell et al., 2000a)*
PKA *(Wright et al., 1997)*	ERK*(Helliwell et al., 2000a)*	ERK *(Helliwell et al., 2000a)*
PKC *(Wright et al., 1997)*	PI3K*(Helliwell et al., 2000a)*	PI3K *(Helliwell et al., 2000a)*
RS1 *(Veyhl et al., 1993)*	mTOR *(Helliwell et al., 2003)*	TNF-α *(Garcia-Herrera, 2004)*
HNF-1 *(Martin et al., 2000)*	AMPK *(Walker et al., 2004)*	
Sp1 *(Martin et al., 2000)*		
Hsp 70 *(Ikari et al., 2002)*		
TGF-β *(Ikari et al., 2002)*		

Table 3. Factors influencing transporter function

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3. HYPOTHESES

My studies were designed to test the following hypotheses:

HYPOTHESIS #1: There is a continuum of decline in intestinal sugar uptake that occurs throughout the lifespan of the healthy rat.

HYPOTHESIS #2: The normal decline in intestinal sugar uptake may be prevented by administering GLP-2 and DEX early in life.

HYPOTHESIS #3: Feeding a diet enriched with saturated as compared to polyunsaturated fatty acids increases intestinal sugar uptake later in life.

HYPOTHESIS #4: The changes in intestinal sugar uptake with age, GLP-2 and DEX, and variations in the type of dietary lipids, are associated with parallel alterations in intestinal morphology and transporter abundance.

HYPOTHESIS #5: The changes in intestinal sugar uptake with age, GLP-2 and DEX, and variations in the type of dietary lipids, are associated with directionally similar alterations in selected signalling proteins.

4. THE AGE-ASSOCIATED DECLINE IN THE INTESTINAL UPTAKE OF GLUCOSE IS NOT ACCOMPANIED BY CHANGES IN THE mRNA OR PROTEIN ABUNDANCE OF SGLT1

4.1. Introduction

The aging of the population has focused attention on the physiological processes associated with aging. The elderly are at a high risk for malnutrition, and while there are many physiological and social factors involved, a reduction in nutrient absorption may contribute to malnourishment. A study using breath hydrogen analysis following a carbohydrate meal showed evidence of malabsorption in the elderly (Feibusch and Holt, 1982). Similarly, transport experiments using isolated brush border membrane (BBM) vesicles demonstrated a reduction in Na+-dependent D-glucose uptake in older persons (Vincenzini et al., 1989). In contrast, a study by Wallis and co-workers (1993) did not find changes in Na+-dependent glucose transport in BBM vesicles isolated from duodenal biopsies from elderly patients (Wallis et al., 1993).

Experiments using rodent models of aging also demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985). Depending on the intestinal site studied, a normal or increased *in vitro* absorptive capacity was also found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption. Ferraris et al. (1993) showed a reduction in uptake and site density of SGLT1 in aged mice (Ferraris and Vinnakota, 1993; Ferraris et al., 1993). This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice (Thompson et al., 1988).

The discrepancies in the results from human, rat and mouse studies may be due to the differences in the methodologies that were used. While some investigators studied uptake using BBM vesicles (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985; Vincenzini et al., 1989; Wallis et al., 1993), others used everted intestinal rings (Thompson et al., 1988; Darmenton et al., 1989; Ferraris and Vinnakota, 1993). As well, the method of expressing results may be important. Most studies have expressed uptake based on intestinal weight, and have therefore not taken into account any potential age-associated changes in mucosal weight or surface area. The strain of the animals used, the ages of the animals, and the site of the intestine used also differ between studies, and may explain the variability in the results.

The sodium-dependent glucose transporter in the brush border membrane (BBM), SGLT1, is responsible for the transport of glucose and galactose into the enterocyte (Wright et al., 1994). The sodium gradient required for SGLT1 activity is maintained by the Na^+K^+-ATPase in the basolateral membrane (BLM) (Horisberger et al., 1991). The uptake of fructose across the BBM is mediated by GLUT5, a sodium-independent facilitative transporter (Thorens, 1996). The transport of glucose, galactose and fructose out of the enterocyte across the basolateral membrane (BLM) occurs via the facilitative sodium-independent GLUT2 transporter (Thorens, 1996). In addition to its role as a BLM transporter, GLUT2 has been localized in the BBM, where it has been suggested to contribute to the uptake of sugars into the enterocyte (Helliwell et al., 2000a,b; Kellett, 2001; Kellett and Helliwell, 2000).

The objectives of this study were to determine (Brasitus et al., 1984) the effects of aging on the *in vitro* uptake of glucose in rats; and (Burant et al., 1994) the molecular mechanisms of these age-associated changes.

4.2. Materials and Methods

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and the Council of the American Physiological Society, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Alberta. Eighteen male Fischer 344 rats, aged 1, 9 and 24 months were obtained from the National Institute of Aging colony and Harlan Laboratories, Maryland, D.C. Pairs of rats were housed at a temperature of

21°C, with 12 hrs of light and 12 hrs of darkness. Water and food were supplied *ad libitum*.

Animals were fed a standard laboratory diet (PMI# 5001). There were a total of 6 animals in each of the three age groups. Animal weights were recorded at weekly intervals.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labelled probes included D-glucose and L-glucose. The labelled and unlabelled probes were supplied by Amersham Biosciences Inc. (Baie d'Urfe, QC) and Sigma (St Louis, MO) respectively. The concentrations used were: D-glucose, 2, 4, 8, 16, 32 and 64 mM; and L-glucose, 16 mM. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume.

Tissue preparation

The animals were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbitol, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with cold saline. The intestine was opened along its mesenteric border, and pieces of the proximal segment (jejunum) and distal segment (ileum) were cut and mounted as flat sheets in the transport chambers. A 5 cm piece of each jejunal and ileal segment gently was scraped with a glass slide to determine the percentage of the intestinal wall comprised of mucosa. The chambers were placed in preincubation beakers containing oxygenated Krebs-bicarbonate buffer (pH 7.2) at 37 °C, and tissue discs were preincubated for 15 min to allow the tissue to equilibrate at this temperature. The rate of uptake of glucose was determined from the timed transfer of the transport chambers to the incubation beakers containing [^{3}H]-inulin and ^{14}C-labelled glucose in oxygenated Krebs-bicarbonate (pH 7.2, 37°C). Preincubation and incubation chambers were mixed with circular magnetic bars at identical stirring rates, which were precisely adjusted using a strobe light. Stirring rates were reported as revolutions per minute (rpm). A stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer

(Lukie et al., 1974; Westergaard and Dietschy, 1974; Westergaard and Dietschy, 1976).

Determination of uptake rates

After incubating the discs in labeled solutions for 6 min, the experiment was terminated by removing the chamber and rinsing the tissue in cold saline for approximately 5 s. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, placed on a glass slide, and dried overnight in an oven at 55°C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Lukie et al., 1974).

The rates of uptake of glucose were determined as nmol 100 mg tissue $^{-1}$ min $^{-1}$, nmol 100 mg mucosal tissue $^{-1}$ min $^{-1}$, nmol cm $^{-2}$ serosal surface area min $^{-1}$, and nmol cm $^{-2}$ mucosal surface area min $^{-1}$. The value of the kinetic constants was determined using a non-linear regression (SigmaPlot 2000, version 6.0), and the reproducibility of values was confirmed using Enzfitter (Version 1.05, Elsevier-Biosoft). Also, linear transformations of the data were plotted using the Lineweaver-Burke plot, the Eadie-Hofstee plot and the Wolfee plot. Statistical significance was accepted for values with $p<0.05$.

Morphology, messenger RNA and protein analysis

Tissue preparation

An additional 12 animals (4 in each of the 3 age groups) were raised and sacrificed similarly as for the uptake studies. A 5 cm portion of proximal jejunum and distal ileum was rinsed, quickly harvested, snap frozen in liquid nitrogen, and stored at −80°C for later mRNA isolation. Mucosal scrapings were harvested from the remaining proximal and distal small intestine, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent isolation of BBM and BLM. For morphological and immunohistochemical analysis, two 1 cm pieces of proximal and distal small intestine were fixed in 10% (w/v) formalin.

Morphological Measurements

Morphometric data were obtained from hematoxylin and eosin stained paraffin sections. Measurements were taken of villous height, villous width at one-half villous height, villous bottom width and crypt depth. Horizontal cross sections were prepared so that villous thickness could be measured at one-half villous height. Magnification was calibrated using a micrometer. Mucosal surface area was calculated as previously described (Thomson et al., 1986). The number of villi per millimeter of serosal length was measured in longitudinal and horizontal cross sections, then multiplied together to obtain the number of villi per square millimeter serosa. When this villous density was multiplied by villous surface area, the result was the mucosal surface area, expressed as square millimeters per square millimeter of serosa. At least 10 villi were assessed per section. The following 2 formulae were used (Thomson et al., 1986):

Villous surface area (μm^2/villus)= $(2 \times M \times H) + (2 \times M–A) \times D + (2 \times D) \times [(A-M)^2 + (H)^2]^{0.5} \times 1000$, where H=villous height, M=villous width at one-half height, A=villous bottom width, and D=villous thickness.

Mucosal surface area (mm^2/mm^2 serosa) = number of villi/mm^2 serosa x villous surface area (μm^2/villus)/1000

Protein analysis

Brush border membranes (BBM), basolateral membranes (BLM), and enterocyte cytosol were isolated from rat intestinal mucosal scrapings by differential centrifugation, and Ca^{2+} precipitation (Orsenigo et al., 1985; Orsenigo et al., 1987; Maenz and Cheeseman, 1986). Aliquots were stored at -80°C.

The protein concentration of the samples was determined using the Bio-Rad Protein Assay® (Life Science Group, Richmond, CA). Proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and were transferred to nitrocellulose membranes.

Transfer efficiency was verified by Ponceau S (3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2, 7-naphtalenediasulfonic acid) staining of membranes, and by Coomassie Blue staining of gels. Membranes were blocked by incubation overnight in BLOTTO (Bovine Lacto Transfer Technique Optimizer) containing 5% w/v dry milk in Tween Tris Buffered Saline (TTBS) (: 0.5% Tween 20, 30mM Tris, 150 mM NaCl).

Membranes were washed in TTBS (3 x 10 min each), and were subsequently probed with specific rabbit anti-rat antibodies to SGLT1 (Chemicon International, Temecula, California), GLUT2 (Biogenesis, Poole, England) and to α1 Na^+K^+-ATPase and β1 Na^+K^+-ATPase (Upstate Biotechnology, Lake Placid, NY). Membranes were incubated for 2 hours at room temperature with antibodies to α1 and β1 Na^+K^+-ATPase. Membranes were incubated overnight at room temperature with SGLT1 antibody. Antibodies were diluted in 2% dry milk in TTBS at 1:500 (SGLT1, GLUT2 and α1 Na^+K^+-ATPase) and 1:1000 (β1 Na^+K^+-ATPase). Membranes were subsequently washed with TTBS to remove the residual unbound primary antibody, and were incubated for 1 hr with goat anti-rabbit antibody (1:20000 in 2 % dry milk in TTBS) conjugated with horseradish peroxidase (HRP) (Pierce, Rockfort, Illinois, USA).

Membranes were washed again in TTBS to remove residual secondary antibody, and were briefly incubated with Supersignal® Chemiluminescent-HRP Substrate (Pierce, Rockfort, Illinois, USA). Membranes were exposed to X-OMAT AR films, and the relative band densities were determined by transmittance densitometry using Bio-Rad Imaging Densitometer (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario). Arbitrary densitometry values are presented as a percentage of the total arbitrary densitometry units for all 3 ages combined.

Immunohistochemistry

Jejunal and ileal tissue was embedded in paraffin, and 4-5 micron sections were mounted on glass slides, dewaxed in xylene, and hydrated after incubation in a series of ethanol incubations. Slides were then placed in hydrogen peroxide/methanol solution (20% - 50% H_2O_2 and 80% methanol) for 6 min, rinsed in tap water, and counterstained with Harris Hematoxylin. Slides were then air-dried, and the tissue was encircled with hydrophobic slide marker (PAP pen, BioGenex, California). After rehydration in phosphate buffered saline (PBS), the slides were incubated for 15 min in blocking reagent (20% normal goat serum), followed by primary antibody to SGLT1 for 30 min. Slides were then washed in PBS and incubated in LINK® and LABEL® according to the manufacturers protocol. The slides were subsequently incubated for 5 min in DAB®, restained in Hematoxylin, rinsed in water, dehydrated in absolute ethanol, and cleared in xylene. Negative controls were processed on the same slide in an identical manner, excluding the incubation with the primary antibody. A Leitz Orthoplan Universal Largefield microscope and a Leitz Vario Orthomat 2 automatic microscope camera were used to photograph the slides. Chromagen staining was quantified using a Pharmacia LKB-Imagemaster DTS densitometer and Pharmacia Imagemaster 1D (Version 1.0) software (Amersham Pharmacia Biotechnology Inc, Piscataway, New Jersey, USA). Villi were divided into 5 equal sections from the villous tip to the crypt, and within each section protein staining was quantified in cells lining the villus in order to determine the distribution of protein along the crypt-villous axis. Four villi per animal were quantified, and the densitometry results were normalized to the negative control values.

Messenger RNA abundance

The intestinal pieces were homogenized in a denaturing solution containing guanidinium thiocyanate, using The Fast Prep® cell disruptor (Savant Instruments Inc., Holbrook, New York.) Following addition of 2 M sodium acetate, a phenol chloroform extraction was performed. The upper aqueous phase containing the RNA was collected. RNA was precipitated with isopropanol overnight at -80ºC, with a final wash with 70% ethanol. The concentration and purity of RNA was determined by spectrophotometry at 260 and 280 nm. Samples were stored at -80ºC until use for Northern blotting, or for reverse transcription polymerase chain reaction (RT-PCR).

Fifteen (15) ug of total RNA was fractionated by agarose gel electrophoresis, and transferred to nylon membranes by capillary diffusion. RNA was fixed to the membrane by baking at 80°C for 2 hr. Northern blotting was performed used the DIG Easy Hyb ® method, according to the manufacturers protocol (Roche Diagnostics, Quebec, Canada).

The α1 and β1 Na^+K^+-ATPase plasmids were generous gifts from Dr J. Lingrel, University of Cincinnati. The SGLT1 plasmid was kindly donated by Dr Davidson, University of Chicago. The GLUT2 plasmid was kindly donated by Dr. G.I. Bell, of the Howard Hughes Medical Institute, University of Chicago.

The density of the mRNA bands was determined by transmittance densitometry (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario). Quantification of the 28 S ribosomal units from the membranes was used to account for loading discrepancies. Arbitrary densitometry units for each age group are presented as a percentage of the total arbitrary densitometry units for all 3 ages combined.

Expression of results

The results were expressed as mean ± standard error of the mean. The statistical significance of the differences between the three age groups was determined by analysis of variance (ANOVA) ($p<0.05$). Individual differences between ages were determined using a Student-Neuman-Keuls multiple range test.

4.3. Results

Animal Characteristics

The rate of body weight change (grams per day) fell between 1, 9 and 24 months (Figure 11). Food intake was not influenced by the age of the rats (data not shown). Age had no effect on the weight of the jejunum, the weight of the scraped jejunal mucosa, or the percentage of the jejunal wall comprised of mucosa (Table 4).

In contrast, the weight of the ileum and the weight of the ileal mucosa were approximately twice as high at 24 as compared with 1 month old animals.

There were no differences in the mean values of the heights of the villi of the jejunum or ileum of rats aged 1, 9 or 24 months (data not shown). There were also no differences in the jejunal or the ileal mucosal surface area at 1, 9 or 24 months (data not shown).

Glucose Absorption

The rates of uptake of 64 mM glucose were first expressed on the basis of the weight of the wall of the intestine (nmol 100 mg^{-1} min^{-1}). There was reduced jejunal and ileal uptake of 64 mM glucose at 9 and 24 months as compared to 1 month (Figure 12). Because the age of the animals influenced mucosal weight (Table 4), glucose uptake was also expressed on the basis of mucosal weight (nmol 100 mg $mucosa^{-1}$ min^{-1}). Again there was reduced jejunal and ileal uptake of glucose between 1 and 9 or 24 months (Figure 13).

When uptake was expressed on the basis of serosal surface area, age and diet had no influence on glucose uptake (data not shown). When the rate of glucose uptake was expressed on the basis of mucosal rather than serosal surface area, the jejunal uptake of glucose was similar at 1, 9 and 24 months, but the ileal uptake was lower at 24 than at 1 or 9 months (Figure 14). Thus, the jejunal and ileal intestinal uptake of glucose is lower in older than in younger rats when expressed on the basis of intestinal weight or mucosal weight, is reduced only in the ileum when expressed on the basis of mucosal surface area, and is not influenced by age when expressed on the basis of serosal surface area.

From the relationship between glucose concentration (2-64 mM) and uptake, the values of the maximal transport rate (Vmax) and Michaelis Menten affinity constant were determined (Tables 5). When the rates of jejunal and ileal glucose uptake were expressed as nmol•100 mg $mucosa^{-1}$• min^{-1} , aging reduced the value of the Vmax by at least 50% between 1 and 24 months, when Vmax was calculated using Sigmaplot, or the Lineweaver-Burke, Eadie Hofstee or Wolfee linear transformation plots (Table 5).

The passive component of D-glucose uptake was estimated from the rate of uptake of L-glucose. In the jejunum, the uptake of L-glucose (nmol 100 mg tissue $^{-1}$ min^{-1}) was similar at 1, 9 and 24 months (Figure 15). In the ileum, the uptake of L-glucose was lower at 9 and 24 months as compared to 1 month.

Transporter Protein Abundance and Immunohistochemistry

The abundance of SGLT1 as determined by Western blotting of the BBM of the jejunum and ileum was similar at 1, 9 and 24 months (Figure 16). For immunohistochemisty, jejunal and ileal villi were divided into 5 equal sections starting from the tip of the villi down to the crypt region. The abundance of SGLT1 protein was evenly distributed along the crypt-villous axis (Figure 20). The jejunal abundance of SGLT1 was increased at 24 months when compared to 1 or 9 months (Figure 17). The ileal abundance of SGLT1 was not affected by age.

The BLM abundance of GLUT2, α1 Na^+K^+ATPase and β1 Na^+K^+ATPase were similar in jejunum and ileum at 1, 9 and 24 months (data not shown).

Transporter mRNA Abundance

There was no significant difference in the jejunal SGLT1 mRNA abundance at 1, 9, and 24 months (Figure 18). In the ileum, SGLT1 mRNA abundance was higher at 9 and 24 months when compared to 1 month.

The jejunal mRNA abundance of β1 Na^+K^+ATPase mRNA was higher at 24 than at 9 months (Figure 19). The jejunal and ileal α1 Na^+K^+ATPase and GLUT2 mRNA abundance was similar at 1, 9 and 24 months of age (data not shown).

4.4. Discussion

In vitro uptake studies allow us to examine the effects of age on nutrient uptake. These results must be substantiated by *in vivo* human data before any conclusions are drawn or recommendations made to the human population. Nonetheless, the *in vitro* studies do help to frame questions that may then be addressed in humans using *in vivo* methods.

With aging, a host of factors may influence nutrient uptake *in vivo*, including alterations in intestinal motility, humoral and neural factors, pancreatic insufficiency, bacterial overgrowth, and the presence of underlying disease, such as diabetes mellitus. *In vitro* uptake studies allow us to specifically investigate the effect of aging on the membrane transport of specific substrates without considering these other factors. This may then lead us to better understand the mechanisms by which nutrient uptake is altered in aging, and to determine if there is indeed a change in uptake kinetics, or if age-associated alterations in nutrient uptake simply reflect changes in other physiological parameters that occur with aging. Furthermore, these studies allow us to address issues of possible mechanisms that might explain age-associated alterations in uptake resulting from variations in intestinal morphology, or alterations in abundance of transporter proteins and their mRNA, or possible variations in the distribution of transporters along the intestinal villous.

The simplest way of expressing the rate of *in vitro* uptake of nutrients is on the basis of the weight of the full thickness of the wall of the intestine. However, if an experimental treatment alters the weight of the intestine or the mucosal surface area, then there may be variations in the rate of nutrient uptake which are understandable in the light of there simply being more mucosal tissue. Three methods of normalizing the data demonstrated an age-associated decline in the uptake of 64 mM glucose (Figures 12-14). Furthermore, using four methods to calculate the value if the Vmax for glucose uptake, aging was associated with a decline in uptake (Table 5). This decline in glucose uptake may have been clinically important, as suggested by the lower body weight gain in the 24 as compared with the 1 or 9 month old rats (Figure 11).

In addition, the changes in *in vitro* glucose uptake seen with aging are not explained by alterations in mRNA and protein abundance of the sugar transporter, SGLT1 (Figures 16,17,20). In fact, the SGLT1 abundance in the jejunum was increased in 24 month old rats when determined by immunohistochemistry. Increases in ileal SGLT1 mRNA with age (Figure 18) do not match the decreased uptake seen with aging, and were not associated with increased ileal abundance of SGLT1

(Figures 16 and 17). In addition, the greater abundance of SGLT1 shown in the jejunum by immunohistochemistry (Figure 17) was not associated with any variations in the abundance of SGLT1 mRNA, and was associated with a decline in glucose uptake (Figures 12-14, Table 5).

The gradient of Na^+ across the BBM is important for the full activity of SGLT1, and this gradient is maintained by the Na^+K^+-ATPase in the BLM (Horisberger et al., 1991). However, the 50% fall in glucose uptake with aging was not associated with a decline in $\alpha1$ or $\beta1$ Na^+K^+-ATPase in the jejunum or ileum (Figures 18 and 19).

In rodent models of diabetes, a "recruitment" of transporters in the lower part of the villi results in active transport occurring in this area, and a resultant increase in glucose transport (Burant et al., 1994). As most glucose uptake occurs in the upper third of the villi (Thomson et al., 1994), a redistribution of protein to this area could explain altered uptake. However, this study did not show changes in the distribution of SGLT1 with age (data not shown). Thus, alterations in the distribution of SGLT1 along the crypt-villus axis could not explain the decline in glucose uptake with aging.

The decline in glucose uptake with aging was not associated with parallel decreases in the mRNA or protein abundance of SGLT1 or Na+K+-ATPase, suggesting that post-translational changes may be occurring that affect the activity of SGLT1. Potential sites for protein kinase A and protein kinase C have been recognized in SGLT1, and binding sites on SGLT1 for glucose are increased by phosphorylation (Ishikawa et al., 1997). The activity of this transporter may therefore, possibly be increased without a concomitant increase in the amount of protein detected by Western blotting and immunohistochemistry.

Changes in the fluidity of the BBM as a result of aging could also explain the apparent uncoupling of transport to mRNA and protein abundance. There are reductions in the membrane fluidity of the BBM isolated from 117 week old Fischer 344 rats as compared to younger animals (Brasitus et al., 1984). Similarly, the fluorescence polarization technique used by Wahnon and coworkers (1989) showed reductions in membrane fluidity in 19 month old rats when compared to 1 and 9 month old rats (Wahnon et al., 1989). Indeed, a study done using chickens demonstrated that reductions in membrane fluidity, as a result of changes in BBM lipid content, may be involved in the decrease in D-glucose uptake observed during post-hatching development (Vazquez et al., 1997). While it is reasonable to speculate that the fluidity of the BBM may have fallen with aging, declines in membrane fluidity are usually associated with increases in uptake (Meddings et al., 1990) rather than the reduction observed in these studies.

It has been proposed that GLUT2 is present in the BBM, as well as in the BLM, and transports glucose into the cell via facilitated diffusion (Kellett, 2001; Helliwell et al., 2000). It is possible that the decline in glucose uptake with aging may have been due to reduced GLUT2 in the BBM of older rats. This speculation cannot be confirmed, as we do not have data for BBM GLUT2 mRNA or protein abundance in the BBM.

In this study three age groups were used to determine if age-related changes in glucose uptake occurred in a step-wise or a continuous manner. When expressed on the basis of intestinal or mucosal weight, uptake was significantly reduced at 9 months of age when compared to 1 month old animals (Figures 12 and 13). No further decrease was seen at 24 months, suggesting that these reductions may be influenced by dietary adaptation in the 1 month old animals or a reflection of a reduced growth rate in the older animals. When expressed on the basis of mucosal surface area, however, ileal uptake is reduced in a step-wise manner, with the decrease only reaching significance in the 24 month old rats (Figure 14). A similar pattern was seen in body weight changes (Figure 11) whereby significant decreases were observed between the 9 and the 24 month old animals. Both of these instances suggest a step-wise change that only becomes apparent in the 24 month old animals. We interpret this to mean that such alterations reflect true age-related changes.

Acknowledgements

We would like to acknowledge Elizabeth Wierzbicki and Terri Canuel for their technical assistance and Dr. Monika Keelan for her invaluable advice regarding the uptake calculations. We also acknowledge the contributions, with thanks, of Arnaud Brulaire, Stephanie Gabet, and Marion Garel. Dr. Gary Wild is a senior clinician scientist supported by the Fonds de la Recherche en Sante du Quebec.

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Table 4. Effect of Age on Intestinal Weight

	1 month	9 months	24 months
Tissue weight (mg/cm)			
Jejunum	9.0±0.7	12.3±1.3	10.7±1.5
Ileum	6.4±0.7	7.8±0.8	11.1±1.7 a
Mucosal weight (mg/cm)			
Jejunum	4.0±0.5	6.3±1.2	5.6±1.1
Ileum	2.9±0.6	3.5±0.6	6.0±1.1 ab
% of Mucosa			
Jejunum	44.5±2.9	48.8±5.2	48.7±4.0
Ileum	38.3±4.7	43.9±3.1	50.1±6.4

Mean ± SEM
a significantly different from 1 month old rats ($p<0.05$)
b significantly different from 9 month old rats ($p<0.05$)

Table 5. Effect of Age on the Values of the Maximal Transport Rate (Vmax) for Glucose Uptake (nmol•100mg mucosal^{-1} min^{-1})

	1 month	9 months	24 months
Jejunum			
SigmaPlot	6848±1254	5232±3224	2522±215 ab
Lineweaver-Burke	2500±0	909±83 a	1000±139 a
Eadie-Hofstee	3533±1.8	1239±96 a	1451±172 a
Wolfee	5000±833	2000±250 a	2000±250 a
Ileum			
SigmaPlot	8415±2668	-----*	1626±328 ab
Lineweaver-Burke	250±67	1111±125	909±115
Eadie-Hofstee	3182±331	1570±233 a	1025±132 a
Wolfee	5000±	2500±417	1429±208

Mean ± SEM
a significantly different from 1 month old rats ($p<0.05$)
b significantly different from 9 month old rats ($p<0.05$)

* not possible to calculate Vmax due to linearity of relationship between glucose concentration and uptake

Table 6. Effect of Age on the Michaelis-Menten affinity constant (Km) Value for Glucose

		1 month	9 months	24 months
Jejunum				
	SigmaPlot	90±24	163±131	46±7
	Lineweaver-Burke	22±5	10±1 a	10±0 a
	Eadie-Hofstee	34.1±7.2	15.1±1.7 a	16.9±0.8 a
	Wolfee	59±17.7	37.4±5	31.2±4.1
Ileum				
	SigmaPlot	96±45	310±301	33±14
	Lineweaver-Burke	1.4±0.2	16.1±2.5 a	9.4±1.1 ab
	Eadie-Hofstee	18±0.9	24±2.4 a	11.2±1.6 ab
	Wolfee	39.5±5.5	2500±233 a	1428±132 ab

Mean ± SEM
a significantly different from 1 month old rats ($p<0.05$)
b significantly different from 9 month old rats ($p<0.05$)

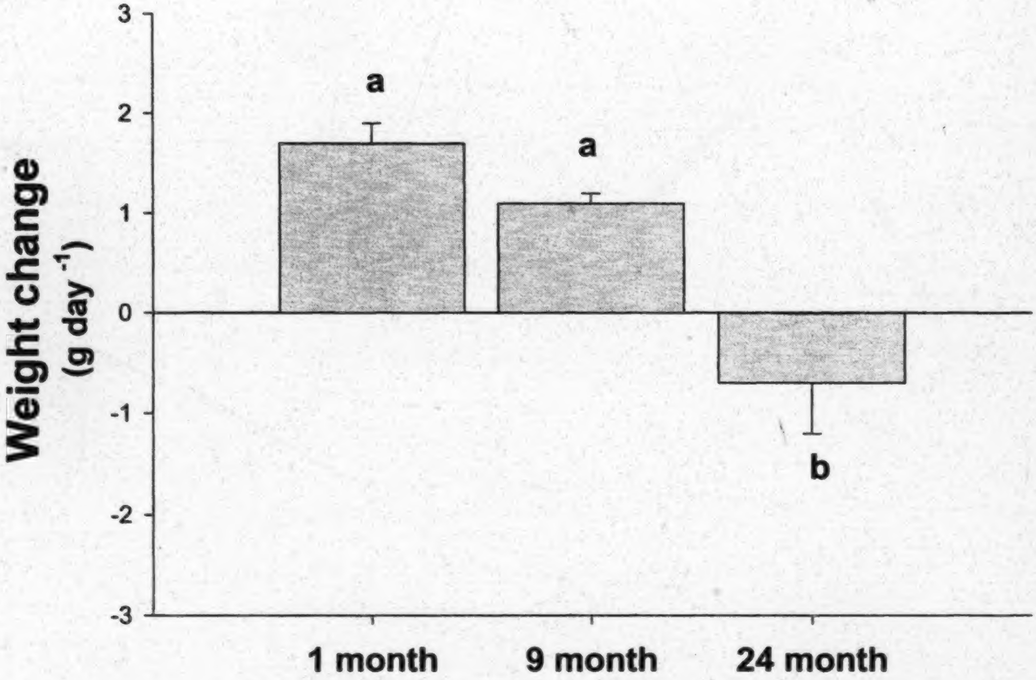
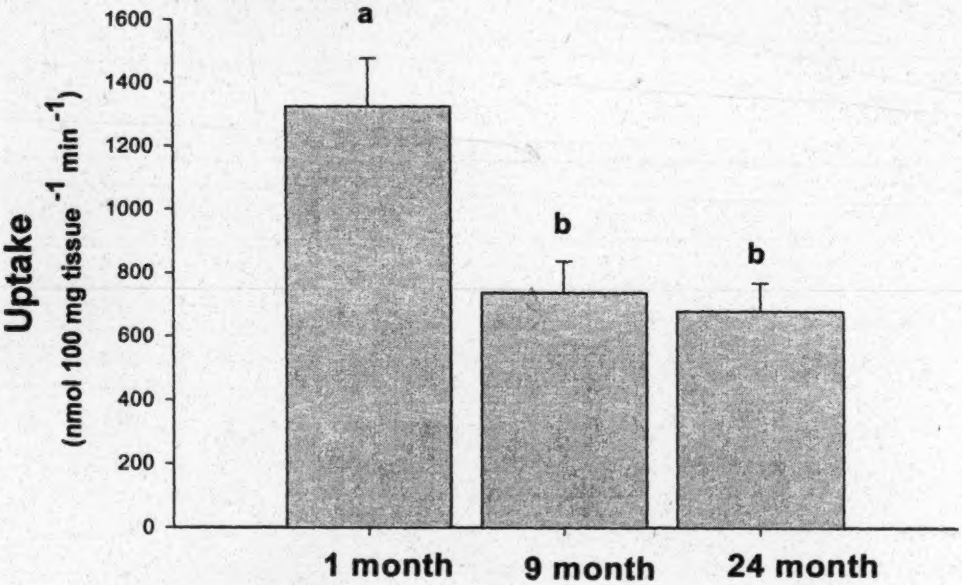


Figure 11. Effect of age on body weight change in F344 rats. Values are Mean ± SEM. Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

(A) Jejunum



(B) Ileum

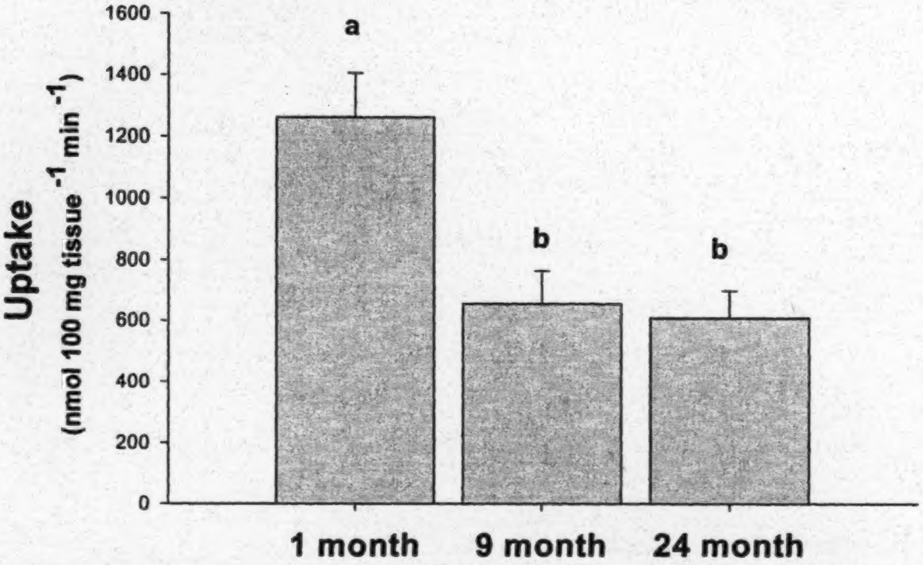
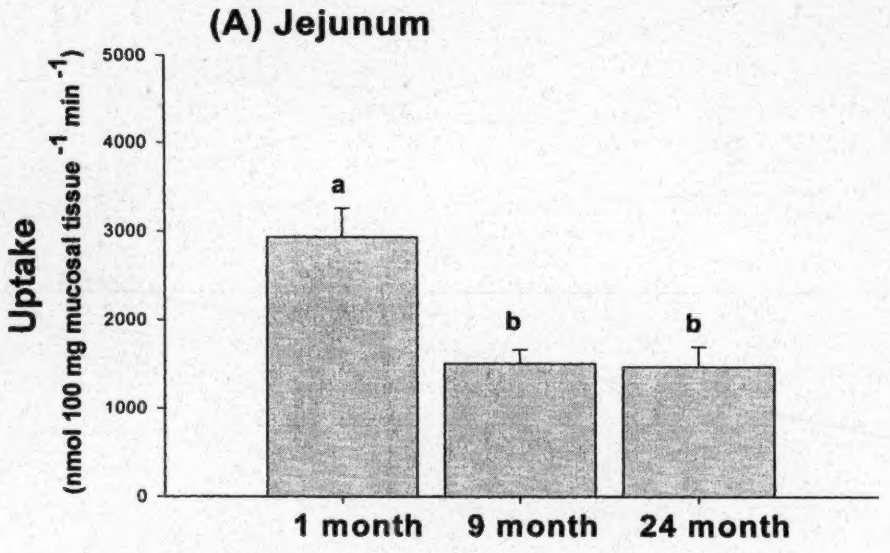


Figure 12. Uptake of 64 mM D-glucose expressed on the basis of intestinal weight in F344 rats. Values are Mean ± SEM Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)



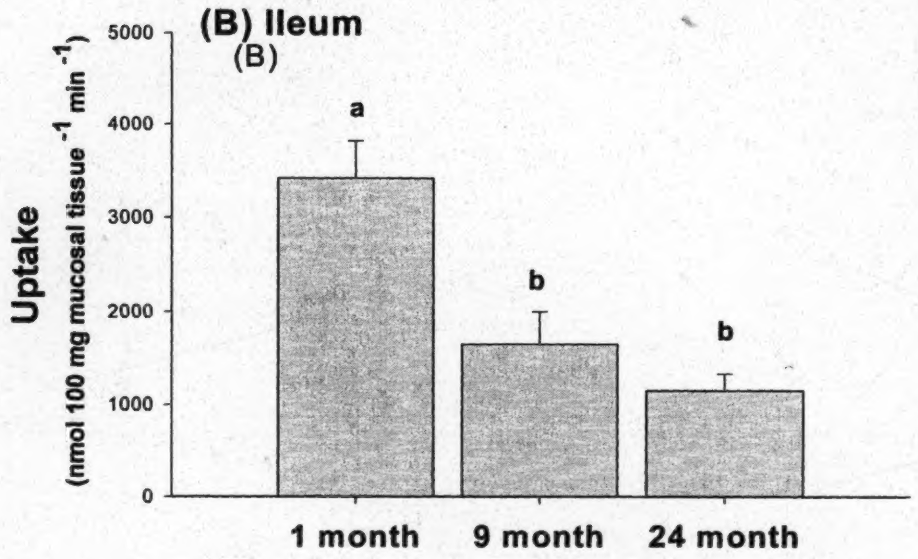
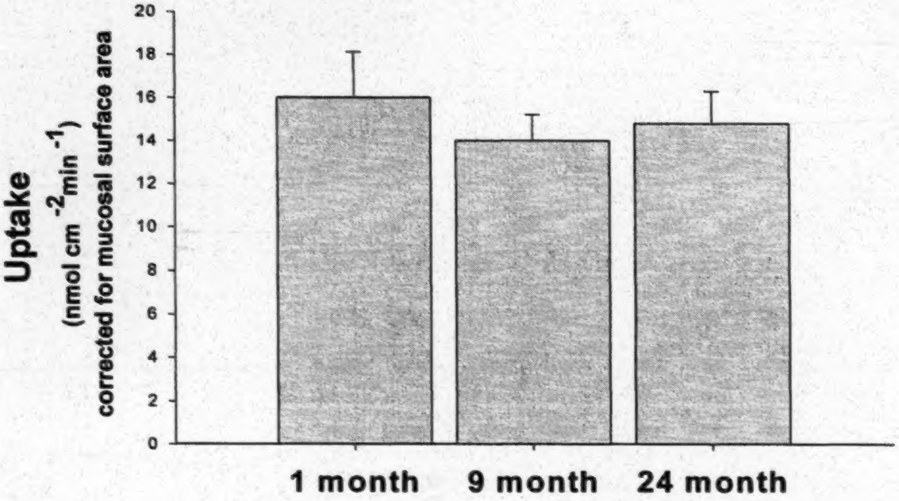


Figure 13. Uptake of 64 mM D-glucose expressed on the basis of mucosal weight in F344 rats. Values are Mean ± SEM. Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

(A) Jejunum



(B) Ileum

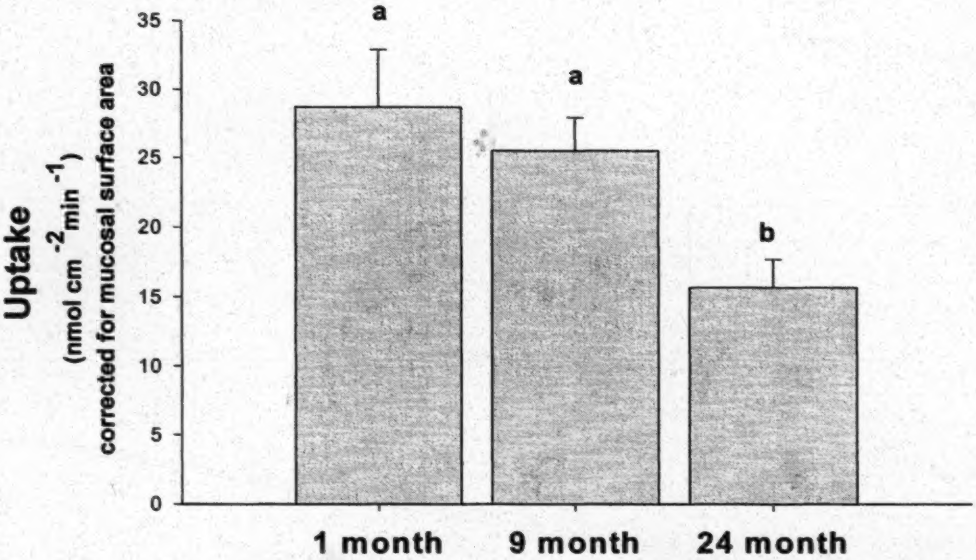
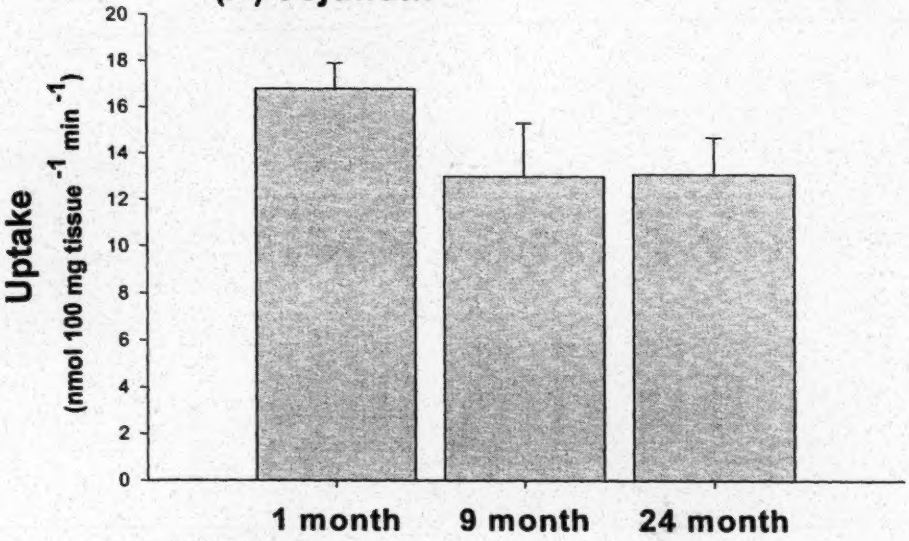


Figure 14. Uptake of 64 mM D-glucose expressed on the basis of mucosal surface area in F344 rats.Values are Mean ± SEM Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)


(A) Jejunum

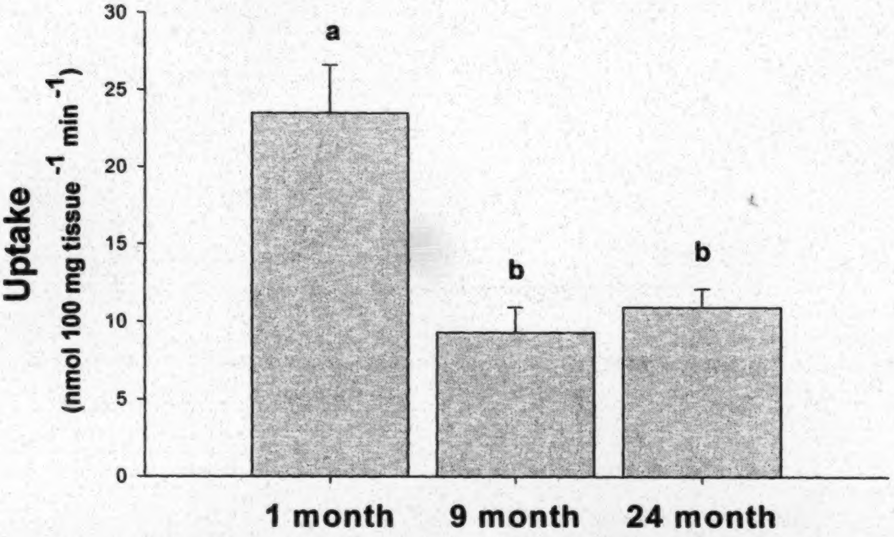

(B) Ileum

Figure 15. Uptake of 16 mM L-glucose expressed on the basis of intestinal weight in F344 rats. Values are Mean ± SEM Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

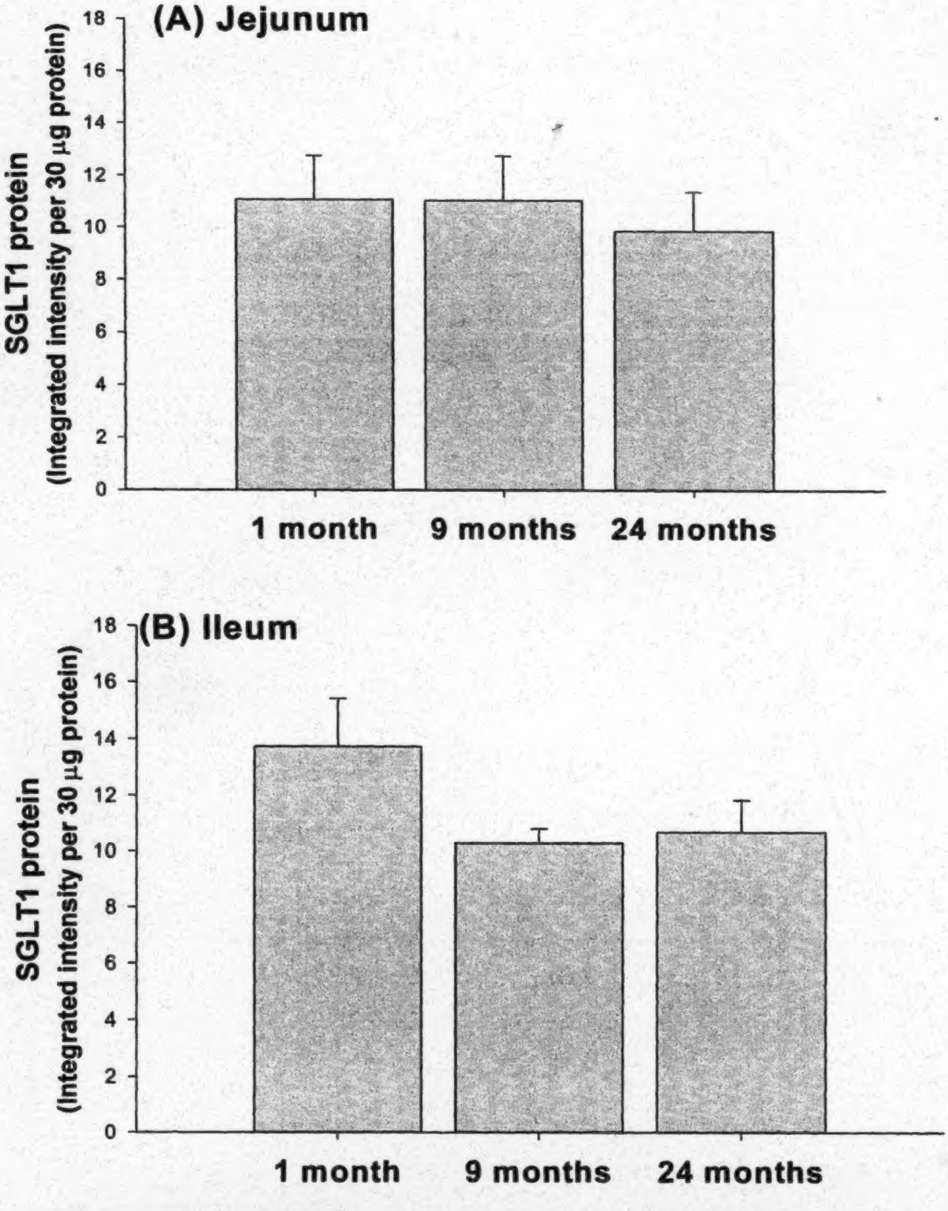


Figure 16. Effect of age on SGLT1 protein abundance in F344 rats
Values are mean ± SEM. None of these differences was statistically significant.

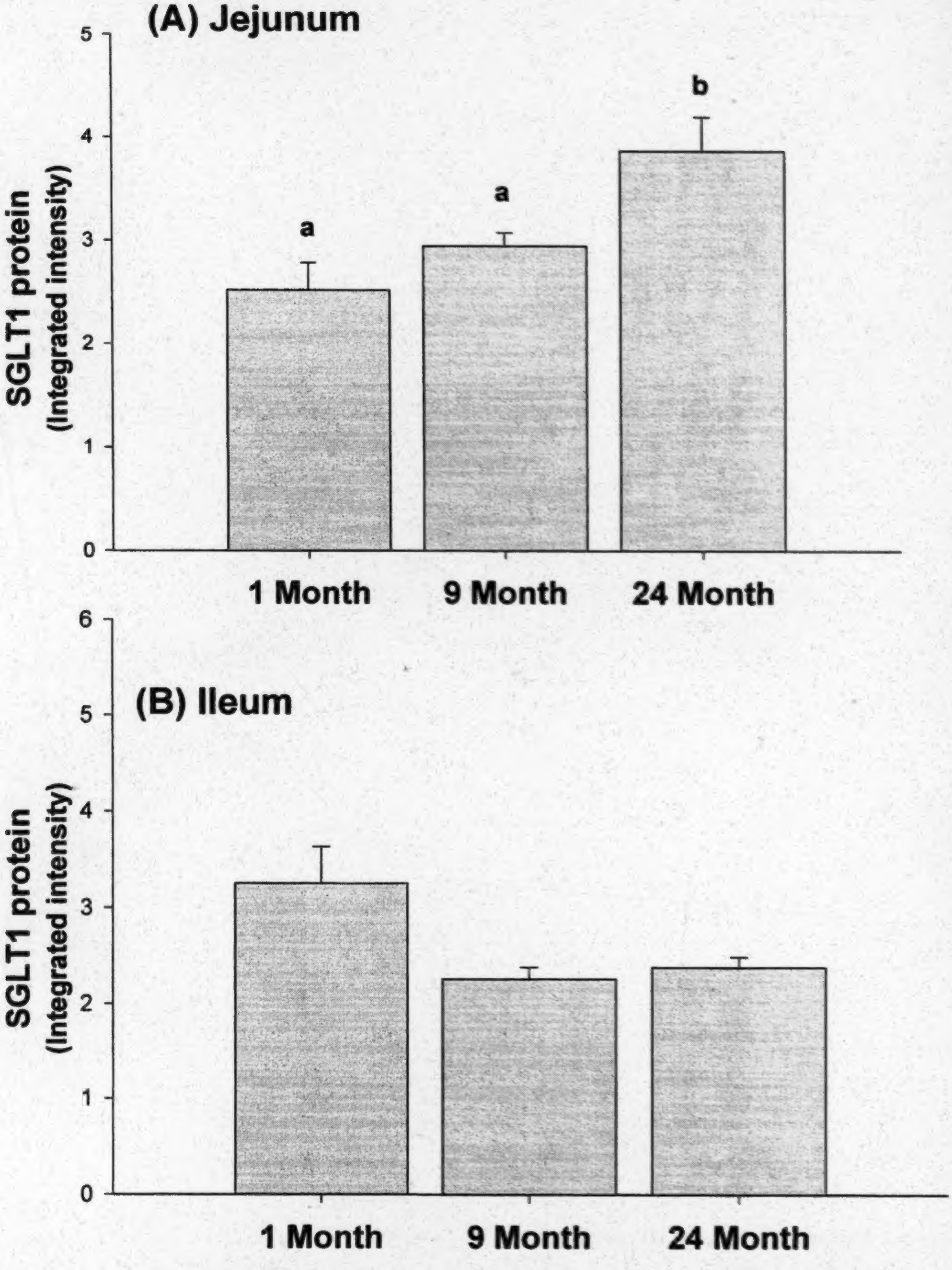


Figure 17. Effect of age on SGLT1 protein abundance as determine by immunohistochemistry in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$).

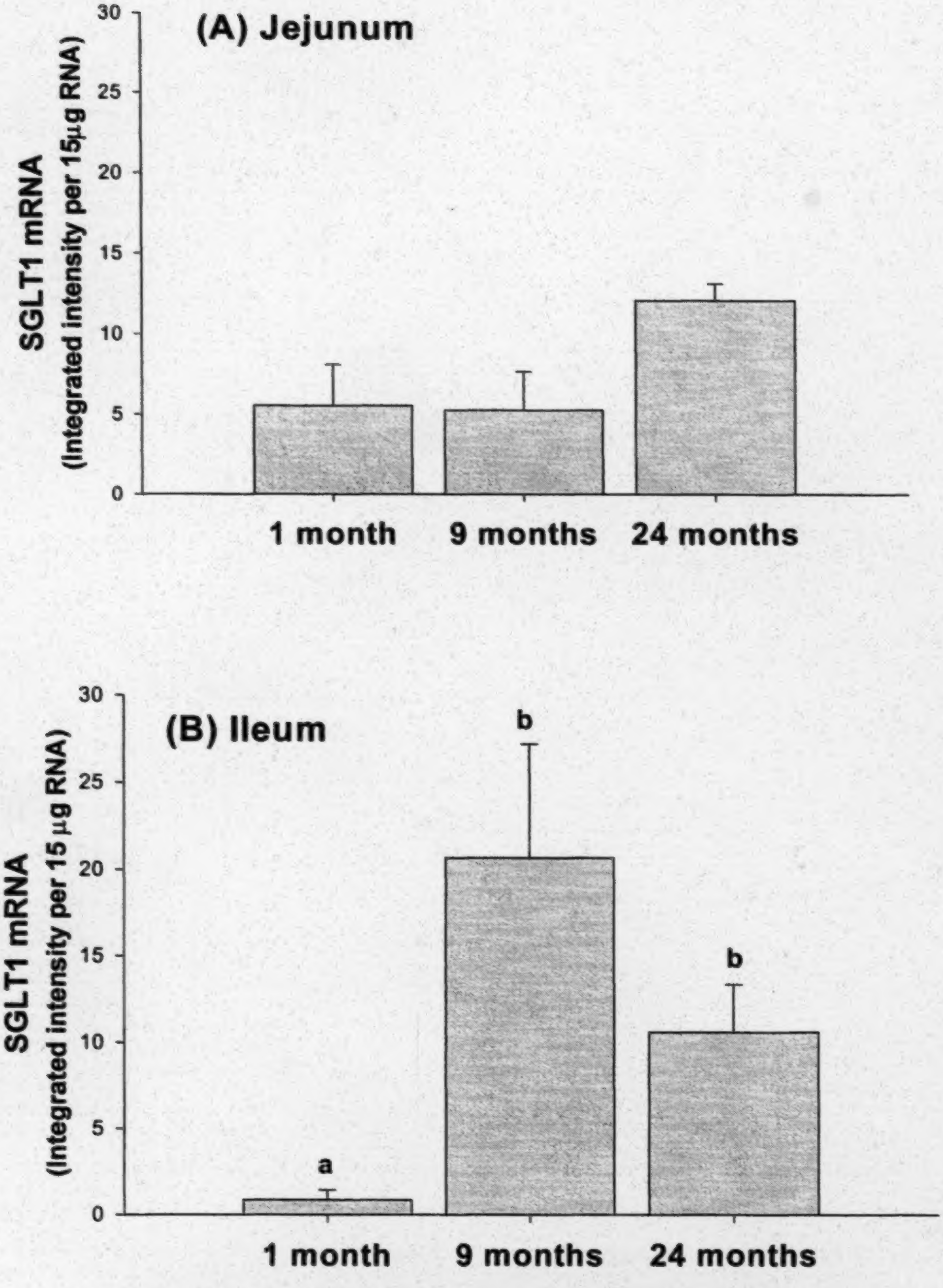


Figure 18. Effect of age on SGLT1 mRNA expression in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$)

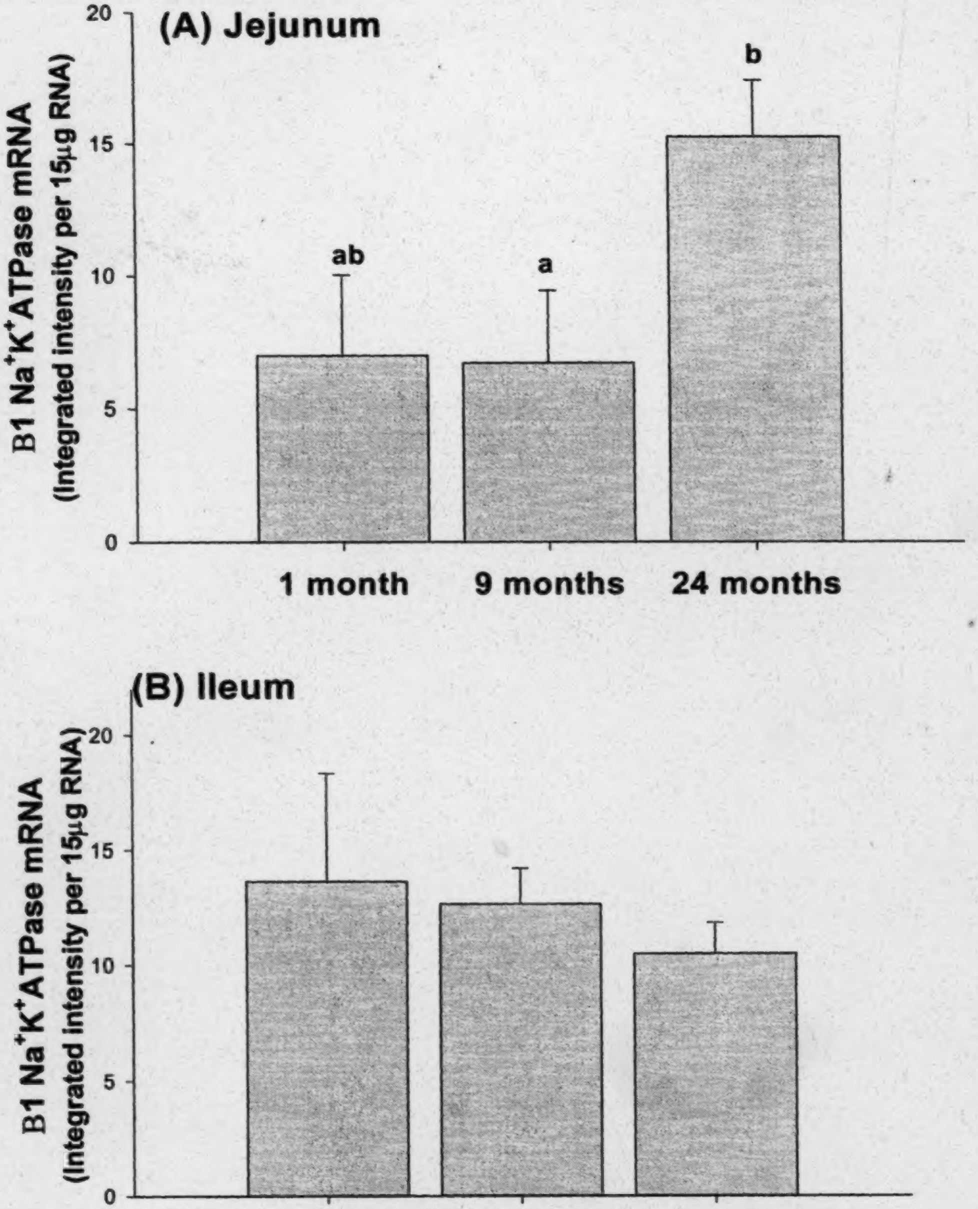


Figure 19. Effect of age on β1 Na^+K^+-ATPase mRNA expression in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$)

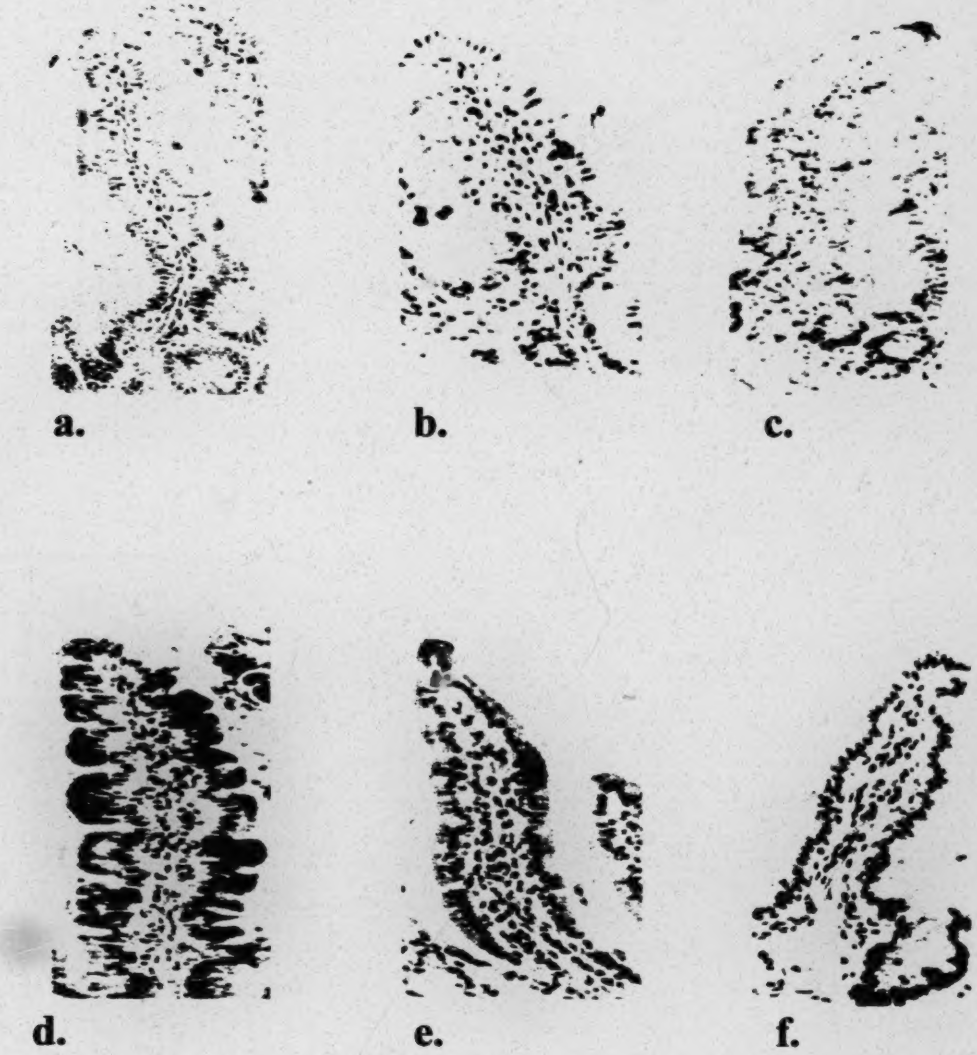


Figure 20. SGLT1 immunohistochemistry on jejunal sections from a) 1 month old rats; b) 9 month old rats; c) 24 month old rats; and ileal sections from d) 1 month old rats; e) 9 month old rats; f) 24 month old rats

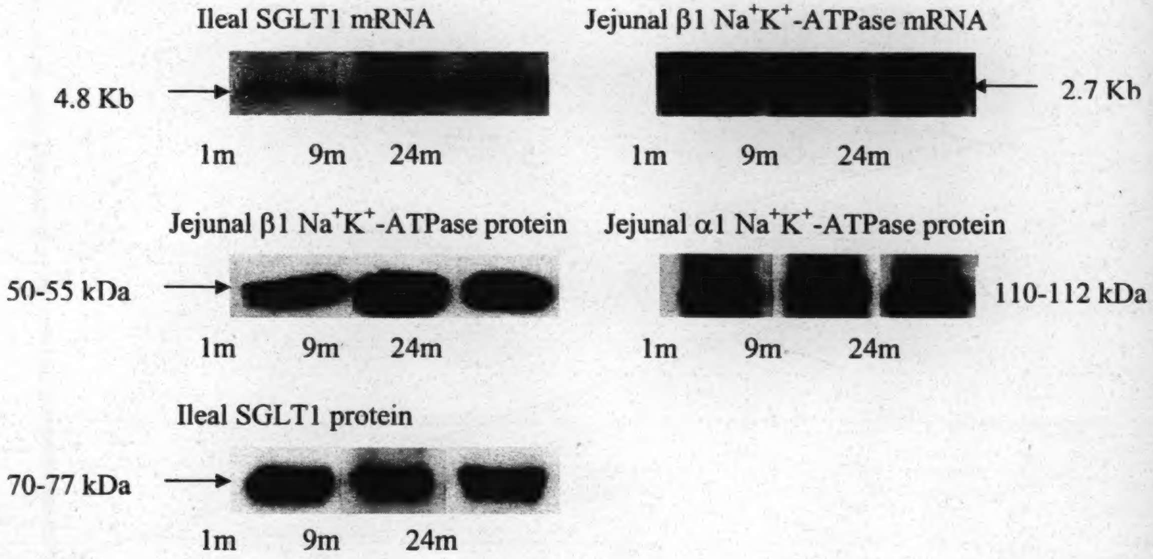

Ileal SGLT1 mRNA
4.8 Kb
1m
9m
24m
Jejunal β1 Na⁺K⁺-ATPase mRNA
2.7 Kb
1m
9m
24m
Jejunal β1 Na⁺K⁺-ATPase protein
50-55 kDa
1m
9m
24m
Jejunal α1 Na⁺K⁺-ATPase protein
110-112 kDa
1m
9m
24m
Ileal SGLT1 protein
70-77 kDa
1m
9m
24m

Figure 21. Representative Northern and Western blots from 1, 9 and 24 month old Fischer 344 rats

4.5. References

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5. AGE-ASSOCIATED CHANGES IN INTESTINAL FRUCTOSE UPTAKE ARE NOT EXPLAINED BY ALTERATIONS IN THE ABUNDANCE OF GLUT5 OR GLUT2

5.1. Introduction

The aging of the population has focused attention on the physiological processes associated with aging. The elderly are at a high risk for malnutrition. While there are many physiological and social factors involved, a reduction in nutrient absorption may contribute to this malnourishment. A study using breath hydrogen analysis following a carbohydrate meal showed evidence of malabsorption in the elderly (Feisbch and Holt, 1982). Similarly, transport experiments using isolated brush border membrane (BBM) vesicles demonstrated a reduction in Na+-dependent D-glucose uptake in older patients (Vincenzini et al., 1989). In contrast, a study by Wallis and co-workers (1993) did not find changes in Na+-dependent glucose transport in BBM vesicles isolated from human duodenal biopsies.

The results from experiments using rodent models of aging also demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Lindi et al., 1985; Freeman and Quamme, 1986; Doubek and Armbrecht, 1987). A normal or increased absorptive capacity, depending on the site studied, was also found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption. Ferraris et al. (1993) show a reduction in uptake and site density of SGLT1 in aged mice. This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice.

The discrepancies in the results from human, rat and mouse studies may be due to the differences in the methodologies that were used. While some investigators study uptake using BBM vesicles (Vincenzini et al., 1989; Wallis et al., 1993; Lindi et al., 1985; Freeman and Quamme, 1986; Doubek and Armbrecht, 1987), others have used everted intestinal rings (Darmenton et al., 1989; Ferraris et al., 1993; Thompson et al., 1988). As well, the method of expressing results is also important. Most studies have expressed uptake based on intestinal weight, and have therefore, failed to take into account any potential age-associated changes in mucosal weight or surface area. The strain of the animals used, the ages of the animals, and the site of the intestine used may also differ between studies, and may explain the variability in results.

The uptake of fructose has been studied in aging mice. Ferraris et al. (1993) showed that D-fructose uptake per milligram of tissue was higher in the jejunum of young as compared to old animals. Adaptive increases in uptake, in response to increasing carbohydrate levels, were blunted in aged mice and were restricted to more proximal regions of the small intestine (Ferraris and Vinnekota, 1993).

The uptake of fructose across the BBM is mediated by GLUT5, a sodium independent facilitative transporter (Thorens, 1996). The transport of fructose and glucose out of the enterocyte across the BLM occurs via the facilitative sodium-independent GLUT2 transporter (Thorens, 1996). In addition to its role as a BLM transporter, GLUT2 has now been localized in the BBM, where it has been suggested to contribute to the uptake of sugars into the enterocyte (Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Kellett, 2000).

The objectives of this study were to determine (1) the effects of aging on the *in vitro* uptake of fructose in rats fed chow; and (2) the molecular mechanisms of these age-associated changes.

5.2. Materials and Methods

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and the Council of the American Physiological Society, were observed in the conduct of this study. Eighteen male Fischer 344 rats, aged 1, 9 and 24 months were obtained from the National Institute of Aging colony and Harlan Laboratories, Maryland, D.C. Pairs of rats were housed at a temperature of 21°C, with 12 hrs of light and 12 hrs of darkness. Water and food were supplied *ad libitum*.

Animals were fed a standard PMI #5001 chow diet. There were a total of 6 animals in each of the three age groups. Animal weights were recorded at weekly intervals.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labelled D-fructose was supplied by Amersham Biosciences Inc. (Baie D'Urfe, Quebec), and the unlabelled fructose by Sigma (St Louis, MO). The concentrations of fructose used were: 8, 16, 32, and 64 mM. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume.

Tissue preparation

The animals were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbitol, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with cold saline. The intestine was opened along its mesenteric border, and pieces of the proximal segment (jejunum) and distal segment (ileum) were cut and mounted as flat sheets in the transport chambers. A 5 cm piece of each jejunal and ileal segment of tissue was gently scraped with a glass slide to determine the percentage of the intestinal wall comprised of mucosa. The chambers were placed in preincubation beakers containing oxygenated Krebs-bicarbonate buffer (pH 7.2) at 37 °C, and tissue discs were preincubated for 15 min to allow the tissue to equilibrate at this temperature. The rate of fructose uptake was determined from the timed transfer of the transport chambers to the incubation beakers containing [^{3}H]-inulin and ^{14}C-labelled fructose in oxygenated Krebs-bicarbonate (pH 7.2, 37°C). Preincubation and incubation chambers were mixed with circular magnetic bars at identical stirring rates, which were precisely adjusted using a strobe light. Stirring rates were reported as revolutions per minute (rpm). A stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer (Lukie et al., 1974; Westergaard and Dietschy, 1974; Westergaard and Dietschy, 1976).

Determination of uptake rates

After incubating the discs in labelled solutions for 6 min, the experiment was terminated by removing the chamber and rinsing the tissue in cold saline for approximately 5 s. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, placed on a glass slide, and dried overnight in an oven at 55°C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Lukie et al., 1974).

The rates of uptake of fructose were determined as nmol 100 mg tissue $^{-1}$ min $^{-1}$, nmol 100 mg mucosal tissue $^{-1}$ min $^{-1}$, and nmol cm $^{-2}$ serosal surface area min $^{-1}$, and nmol cm $^{-2}$ villus surface area min $^{-1}$. Because the relationship between uptake and fructose concentration was linear, the slopes of the lines were calculated and compared to determine statistical differences. Statistical significance was accepted for values with $p<0.05$.

Morphology, mRNA and protein analysis

Tissue preparation

An additional 12 animals (4 in each of the 3 age groups) were raised and sacrificed similarly as for the uptake studies. A 5 cm portion of proximal jejunum and distal ileum was rinsed, quickly harvested, snap frozen in liquid nitrogen, and stored at –80°C for subsequent mRNA isolation. Mucosal scrapings were harvested from the remaining proximal and distal small intestine, snap-frozen in liquid nitrogen, and stored at –80°C for later isolation of cellular components. For morphology and immunohistochemistry analysis, two 1cm pieces of proximal and distal small intestine were fixed in 10 % formalin.

Morphological Measurements

Morphometric data were obtained from hematoxylin and eosin stained paraffin sections. Measurements were taken of villous height, villous width at one-half villous height, villous bottom width and crypt depth. Horizontal cross sections were prepared so that villous thickness could be measured at one-half villous height. Magnification was calibrated using a micrometer. Mucosal surface area was calculated as previously described (Thomson et al., 1986). The number of villi per millimeter of serosal length was measured in longitudinal and horizontal cross sections, then multiplied together to obtain the number of villi per square millimeter serosa. When this villous density was multiplied by villous surface area, the result was the mucosal surface area, expressed as square millimeters per square millimeter of serosa. At least 10 villi were assessed per section. The following 2 formulae were used (Thomson et al., 1986):

Villous surface area (μm^2/villus)= $(2 \times M \times H) + (2 \times M–A) \times D + (2 \times D) \times [(A-M)^2 + (H)^2]^{0.5} \times 1000$, where H=villous height, M=villous width at one-half height, A=villous bottom width, and D=villous thickness.

Mucosal surface area (mm^2/mm^2 serosa) = number of villi/mm^2 serosa x villus surface area (μm^2/villus)/1000

mRNA abundance

The intestinal pieces were homogenized in a denaturing solution containing guanidinium thiocyanate, using The Fast Prep® cell disruptor (Savant Instruments Inc., Holbrook, New York.) Following addition of 2 M sodium acetate, a phenol chloroform extraction was performed. The upper aqueous phase containing the RNA was collected. RNA was precipitated with isopropanol overnight at -80°C, with a final wash with 70% ethanol. The concentration and purity of RNA was determined by spectrophotometry at 260 and 280 nm. Samples were stored at -80°C until use for Northern blotting, or for RT-PCR (reverse transcriptase- polymerase chain reaction.)

Fifteen (15) ug of total RNA was fractionated by agarose gel electrophoresis and transferred to nylon membranes by capillary diffusion. RNA was fixed to the membranes by baking at 80°C for 2 hr. Northern blotting was performed used the DIG Easy Hyb ® method, according to the manufacturers protocol (Roche Diagnostics, Quebec, Canada).

The GLUT2 and GLUT5 plasmids were kindly donated by Dr. G.I. Bell, of the Howard Hughes Medical Institute, University of Chicago.

The density of the mRNA bands was determined by transmittance densitometry (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario). Quantification of the 28 S ribosomal units from the membranes was used to account for loading discrepancies.

Protein analysis

Brush border membranes (BBM), basolateral membranes (BLM), and enteroctye cytosol were isolated from rat intestinal mucosal scrapings by differential centrifugation, and Ca^{2+} precipitation (Maenz and Cheeseman, 1986; Orsenigo et al., 1985; Orsenigo et al., 1987). Aliquots were stored at -80°C.

The protein concentration of the samples was determined using the Bio-Rad Protein Assay® (Life Science Group, Richmond, CA). Proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and were transferred to nitrocellulose membranes by electroblotting.

Transfer efficiency was verified by Ponceau S (3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2, 7-naphtalenediasulfonic acid) staining of membranes, and by Coomassie Blue staining of gels. Membranes were blocked by incubation overnight in BLOTTO (Bovine Lacto Transfer Technique Optimizer) containing 5% w/v dry milk in Tween Tris Buffered Saline (TTBS) (: 0.5% Tween 20, 30mM Tris, 150 mM NaCl).

Membranes were washed in TTBS (3 x 10 min each), and were subsequently probed with specific rabbit anti-rat antibodies to GLUT5 (Chemicon International, Temecula, California) and GLUT2 (Biogenesis, Poole, England). Membranes were incubated for 2 hours at room temperature with the antibodies. Antibodies were diluted in 2% dry milk in TTBS at 1:500. Membranes were subsequently washed with TTBS to remove the residual unbound primary antibody, and were then incubated for one hr with goat anti-rabbit antibody (1:20000 in 2 % dry milk in TTBS) conjugated with horseradish peroxidase (HRP) (Pierce, Rockfort, Illinois, USA).

Membranes were washed again in TTBS to remove residual secondary antibody, and were briefly incubated with Supersignal® Chemiluminescent-HRP Substrate (Pierce, Rockfort, Illinois, USA). Membranes were exposed to X-OMAT AR films, and the relative band densities were determined by transmittance densitometry using Bio-Rad Imaging Densitometer (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario).

Immunohistochemistry

Jejunal and ileal tissue was embedded in paraffin, and 4-5 micron sections were mounted on glass slides, dewaxed in xylene, and hydrated after incubation in a series of ethanol incubations. Slides were then incubated in hydrogen peroxide/methanol solution (20% - 50% H_2O_2 and 80% methanol) for 6 min, rinsed in tap water and counterstained with Harris Hematoxylin. Slides were then air-dried and the tissue was encircled with hydrophobic slide marker (PAP pen, BioGenex, California). After rehydration in phosphate buffered saline (PBS), the slides were incubated for 15 min in blocking reagent (20% normal goat serum) followed by primary antibody to GLUT5 for 30 min. Slides were then washed in PBS and incubated in LINK® and LABEL® according to the manufacturers protocol. The slides were subsequently incubated for 5 min in DAB® solution rinsed in water, dehydrated in absolute ethanol, and cleared in xylene. Negative controls were processed on the same slide in an identical manner, excluding the incubation with the primary antibody. A Leitz Orthoplan Universal Largefield microscope and a Leitz Vario Orthomat 2 automatic microscope camera were used to photograph the slides. Chromagen staining was quantified using a Pharmacia LKB-Imagemaster DTS densitometer and Pharmacia Imagemaster 1D (Version 1.0) software (Amersham Pharmacia Biotechnology Inc, Piscataway, New Jersey, USA). Four villi per animal were quantified, and the results were normalized to the negative control values.

Expression of results

The results were expressed as mean ± standard error of the mean. The statistical significance of the differences between the three age groups was determined by analysis of variance (ANOVA) ($p<0.05$). Individual differences between ages were determined using a Student-Neuman-Keuls multiple range test. Sigmastat (version 1) statistical prgaram was used for all statistical analysis.

5.3. Results

Animal Characteristics

The rate of body weight change (grams per day) was significantly lower at 24 months when compared to 1 or 9 month old animals (Figure 22). Food intake was not influenced by the age of the rats (data not shown). Age did not affect the weight of the jejunum, the weight of the scraped jejunal mucosa, or the percentage of the jejunal wall comprised of mucosa (Table 7). In contrast, the weight of the ileum and the weight of the scraped ileal mucosal were approximately twice as high at 24 months when compared to 1 month old animals.

There were no differences in the mean values of the heights of the villi of the jejunum or ileum of rats aged 1, 9 or 24 months (data not shown). Similarly, there were no differences in the jejunal or the ileal mucosal surface area at 1, 9 or 24 months (data not shown).

Fructose Absorption

When fructose uptake was expressed on the basis of the weight of the entire wall of the intestine (nmol 100 mg tissue^{-1} min^{-1}), there was reduced jejunal fructose uptake between 1 and 9 but not between 1 and 24 months, whereas the ileal uptake of fructose was reduced at both 9 and 24 months (Figure 23). When the rate of uptake was expressed on the basis of the weight of the mucosa (nmol 100mg mucosal tissue^{-1} min^{-1}), fructose uptake into the jejunum was lower at 9 and 24 months as compared to 1 month, and uptake into the ileum was lower at 9 months, but not at 24 months as compared to 1 month (Figure 24). When uptake was expressed on the basis of serosal surface area, fructose uptake in the jejunum was higher at 24 than at 9 or at 1 month, and was unchanged by age in the ileum (Figure 25). When uptake was expressed on the basis of mucosal surface area, jejunal fructose uptake was higher at 24 than at 9 or at 1 month, whereas ileal uptake was unaffected by age (Figure 26). When the slopes of fructose uptake were compared, a significant decrease was seen at 9 and 24 months when compared to 1 month old animals in both the jejunum and the ileum (Table 8).

Transporter Protein Abundance and Immunohistochemistry

The jejunal abundance of GLUT5 in the BBM, as determined by Western blotting, was higher at 24 as compared with 1 month (Figure 27). The BBM abundance of GLUT5 in the ileum was similar at 1, 9 and 24 months of animals. For immunohistochemistry, jejunal and ileal villi were divided into 5 equal sections starting from the tip of the villi down to the crypt region. The abundance of GLUT5 protein was evenly distributed along the crypt-villous axis (Figure 28). The jejunal abundance of GLUT5 was increased with aging (Figure 29). In the ileum, GLUT5 was also increased at both 9 and 24 months when compared to 1 month old animals.

The abundance of GLUT2 in the BLM was similar in 1, 9 and 24 month old animals (data not shown).

Transporter mRNA abundance

The mRNA abundance of GLUT2 mRNA in the jejunum was similar in 1, 9 and 24 month old animals (data not shown).

5.4. Discussion

The simplest way of expressing the rate of *in vitro* uptake of nutrients is on the basis of the weight of the full thickness of the wall of the intestine. However, if a treatment alters the weight of the intestine, then there may be variations in the rate of nutrient uptake which are understandable in the light of there simply being more mucosal tissue. This may be an appropriate method of expressing the results of the uptake data when attempting to answer the question "Does aging affect fructose absorption?", but does not provide an explanation of the mechanisms of the age-associated alterations. For this reason, where there are treatment-associated variations in mucosal mass or the surface area of the villous membrane, then it is more appropriate to express uptake on the basis of mass of the transporting mucosal tissue or the mucosal surface area. When this was taken into consideration, aging was associated with a reduction in fructose uptake when expressed as intestinal or mucosal weight (Figures 23 and 24), whereas aging was associated with increased jejunal fructose uptake when expressed as serosal or mucosal surface area (Figures 25 and 26). Thus, the direction of the effect of aging on fructose uptake depends on the method used to express the rates of uptake.

The changes in fructose uptake seen with aging are not explained by alterations in mRNA and protein abundance of the fructose transporters. At 24 months of age, the increase in jejunal fructose uptake expressed as serosal or mucosal surface (Figures 25 and 26) is paralleled by increases in the abundance of GLUT5, as determined by Western blotting and by immunohistochemistry (Figures 27 and 29). However, this does not apply when fructose uptake is expressed on the basis of intestinal or mucosal weight. In the ileum, between 24 and 1 month of age fructose uptake either falls (Figure 23) or is unchanged (Figures 24-26), depending upon how the data is expressed, and yet GLUT5 abundance is unchanged (Figure 27) or increases (Figure 29). Thus, the age-associated changes in the uptake of fructose is either associated with no change or an increase in the abundance of GLUT5 in the BBM.

In models of diabetes, a "recruitment" of transporters in the lower part of the villi results in active transport occurring in this area, and a resultant increase in glucose transport (Burant et al, 1985). Changes in the distribution of GLUT5 along the crypt villus axis might also explain changes in glucose uptake with aging. As most intestinal uptake occurs in the upper third of the villi (Thomson et al., 1994), a redistribution of transporter protein to this area may explain altered uptake. The results from this study do not show changes in the distribution of GLUT5 with age (data not shown), and thus it is unlikely that the age-associated changes in fructose uptake are due to the premature activation of transporters along the crypt-villous axis.

Changes in the fluidity of the BBM as a result of aging could also explain the apparent uncoupling of transport to gene abundance and protein abundance. There are reductions in the membrane fluidity of the BBM isolated from 117 week old Fischer 344 rats as compared to younger animals (Brasitus et al., 1984). Similarly, the fluorescence polarization technique used by Wahnon et al. (1989) showed reductions in membrane fluidity in 19 month old rats when compared to 1 and 9 month old rats. Indeed, a study using chickens demonstrated that changes in BBM lipid content and fluidity may be involved in the alterations observed in D-glucose uptake during post-hatching development (Vazquez et al., 1997). While it is reasonable to speculate that the fluidity of the BBM may have fallen with aging, declines in membrane fluidity are associated with increases in uptake (Meddings et al., 1990) rather than the variable results seen in this study.

It has been proposed that GLUT2 is present in the BBM as well as in the BLM (Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Kellett, 2000) and transports glucose and fructose into the cell via facilitated diffusion. The changes in fructose uptake with aging may therefore be paralleled by alterations in BBM GLUT2 protein. This speculation cannot be confirmed in this study, as we do not have data for BBM GLUT2 protein abundance.

Other investigators have demonstrated that GLUT2 may not be essential for sugar absorption. In GLUT2 deficient mice (RIPGLUT1xGLUT2-/-), an oral glucose load still resulted in a normal rate of glucose appearance in blood, suggesting that GLUT2 is not required (Thorens et al., 2000). Similarly, Stumpel et al. (2001) demonstrated identical kinetics of transepithelial glucose transport in GLUT2 deficient mice, when compared to control mice following oral glucose tolerance tests.

In summary, the results indicate that the effect of age on fructose uptake depends on the method used to express results. Although it may appear that fructose uptake is reduced with age, when reductions in mucosal surface area are taken into account, fructose uptake increases (jejunum) or is unchanged (ileum) with age. Finally, the age-associated changes in uptake are not fully explained by alterations in GLUT5 and GLUT2.

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Figure 29 Representative images of GLUT5 immunohistochemistry

Table 7. Effect of Age on Intestinal Weight

	1 month	9 months	24 months
Tissue weight (mg/cm)			
Jejunum	9.0±0.7	12.3±1.3	10.7±1.5
Ileum	6.4±0.7 a	7.8±0.8 ab	11.1±1.7 b
Mucosal weight (mg/cm)			
Jejunum	4.0±0.5	6.3±1.2	5.6±1.1
Ileum	2.9±0.6 a	3.5±0.6 a	6.0±1.1 b
% of Mucosa			
Jejunum	44.5±2.9	48.8±5.2	48.7±4.0
Ileum	38.3±4.7	43.9±3.1	50.1±6.4

Mean ± SEM
Different letters indicate a statistically significant age effect ($p<0.05$)

Table 8. Slopes for D-fructose uptake in the jejunum and ileum of Fischer 344 rats

SITE	**AGE**	**SLOPE**
Jejunum	1 month	44.4±2.4 a
	9 months	25.5±3.0 b
	24 months	31.4±1.1 b
Ileum	1 month	51.4±2.7 a
	9 months	26.8±4.0 b
	24 months	19.5±0.9 b

Mean ± SEM
Different letters indicate a statistically significant age effect ($p<0.05$)

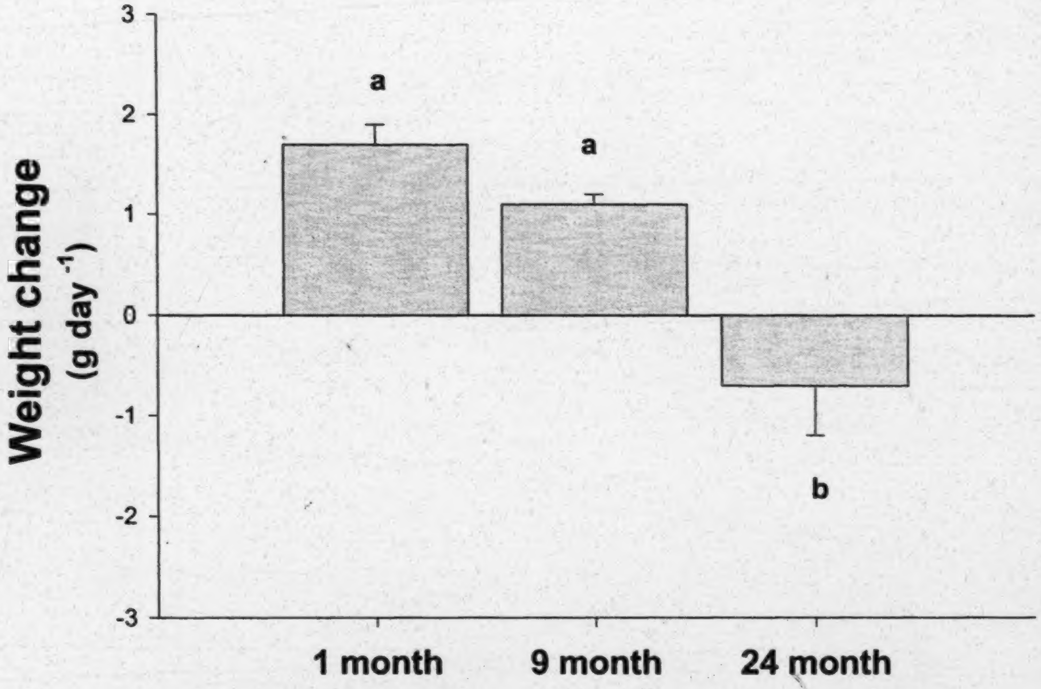


Figure 22. Effect of age on body weight change in F344 rats. Values are Mean ± SEM. Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

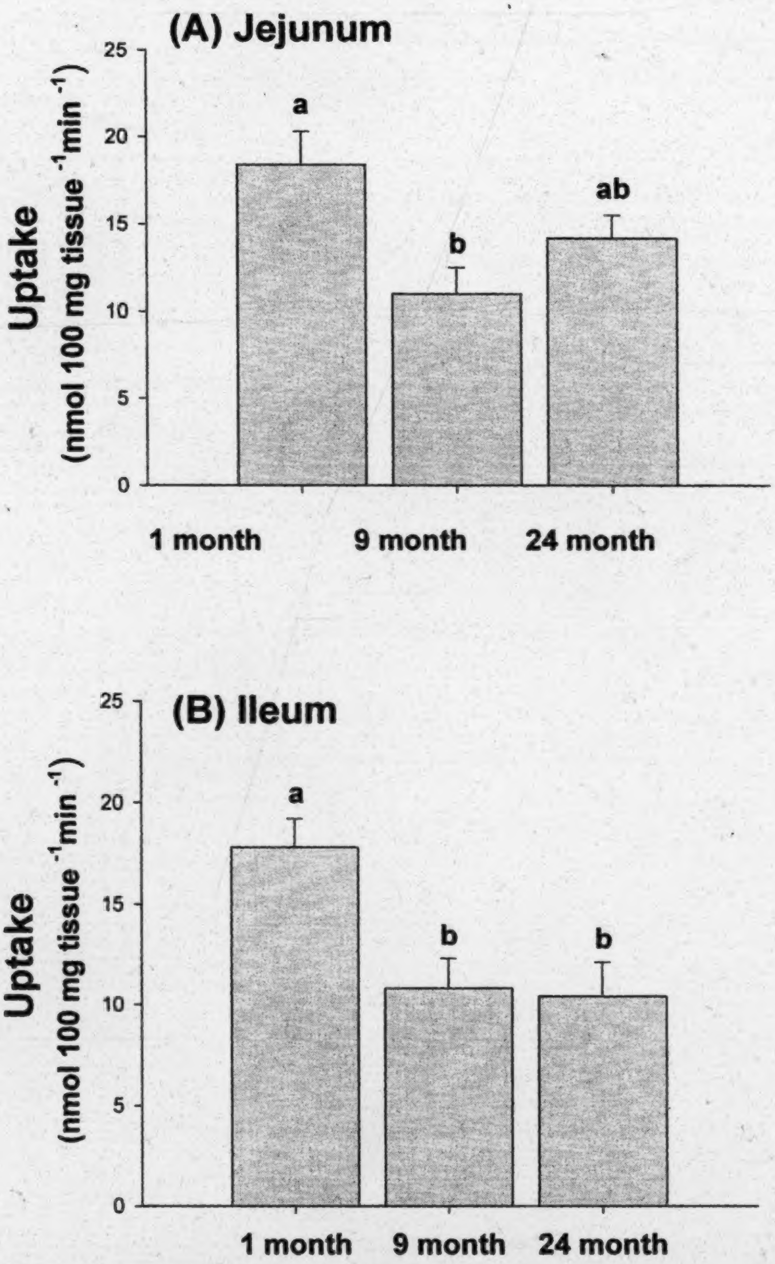


Figure 23. Uptake of D-fructose expressed on the basis of intestinal weight in F344 rats. Values are Mean ± SEM of the slope of the linear regression as calculated using SigmaPlot. Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

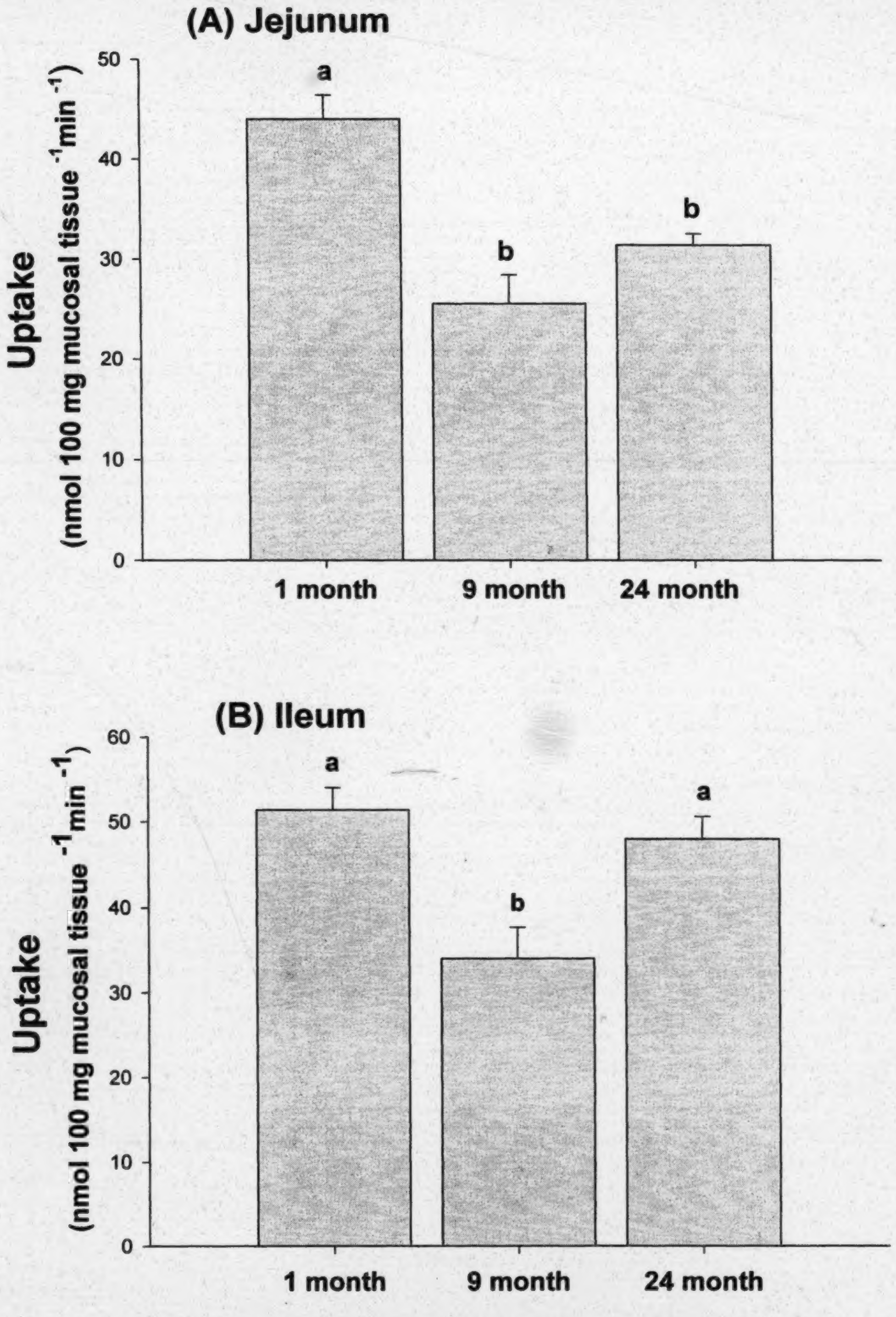


Figure 24. Uptake of D-fructose expressed on the basis of mucosal weight in F344 rats. Values are Mean ± SEM of the slope of the linear regression as calculated using SigmaPlot.
Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

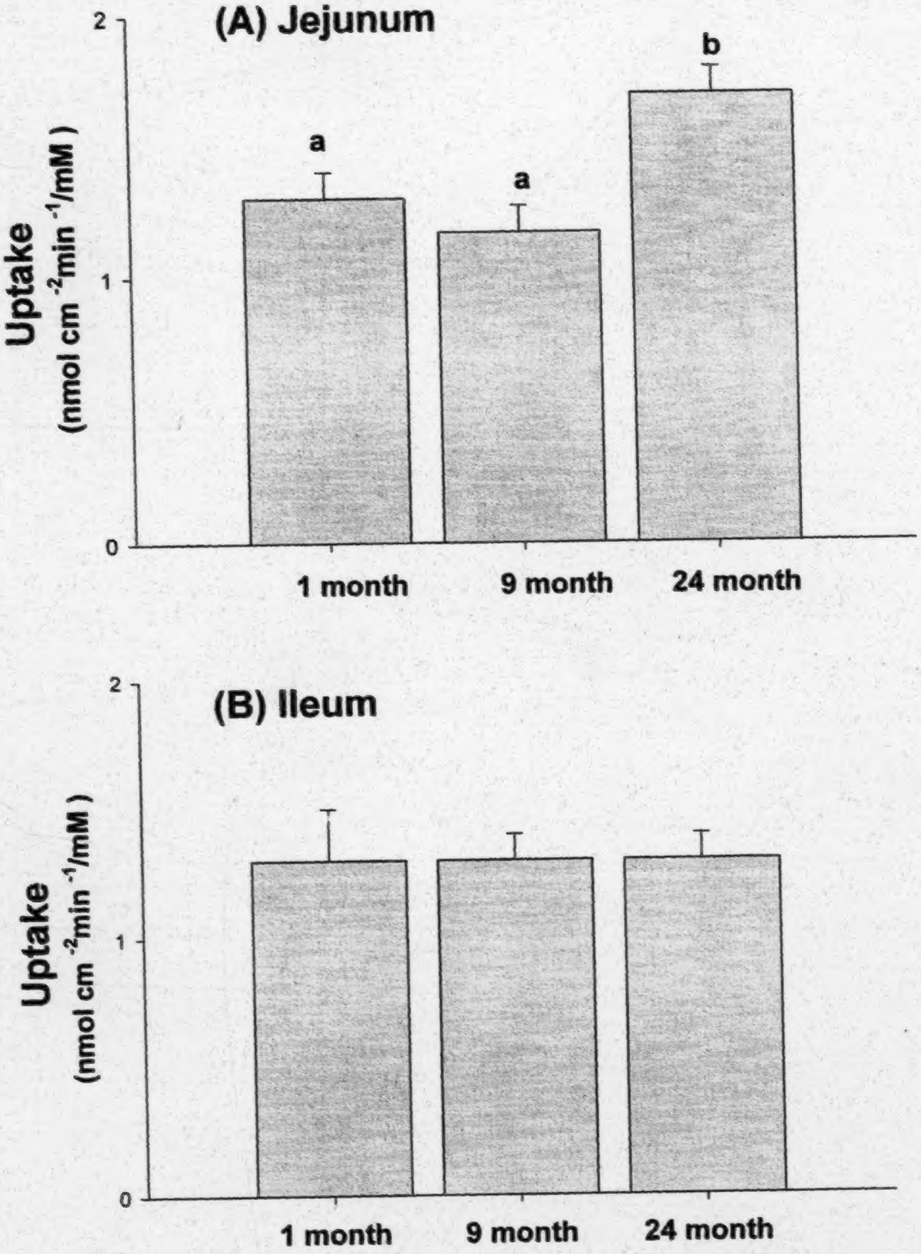
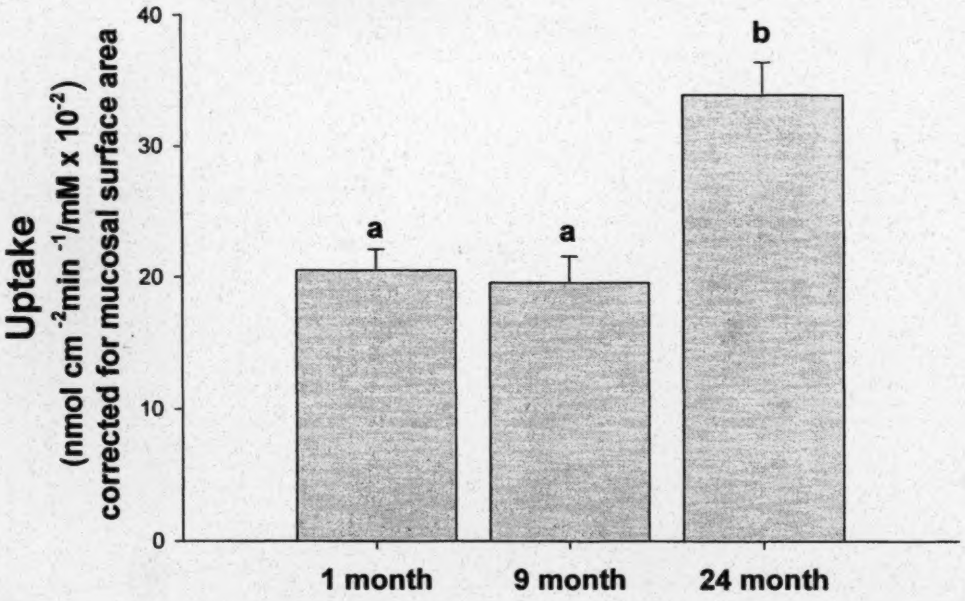


Figure 25. Uptake of D-fructose expressed on the basis of serosal surface area in F344 rats. Values are Mean ± SEM of the slope of the linear regression as calculated using SigmaPlot.
Different letters indicate significant age effect (ANOVA $p \leq 0.05$)

(A) Jejunum



(B) Ileum

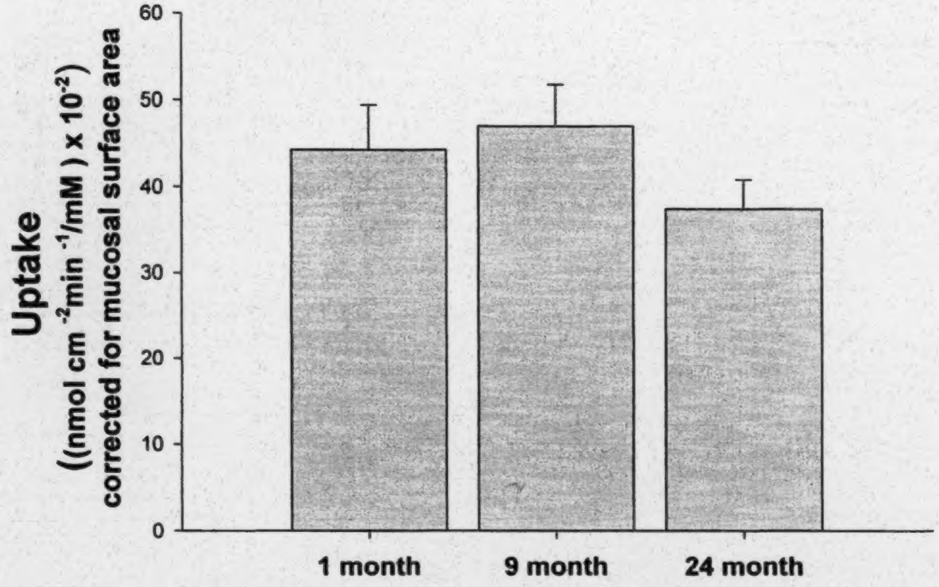


Figure 26. Uptake of D-fructose expressed on the basis of mucosal surface area in F344 rats.Values are Mean + SEM of the slope of the linear regression as calculated using SigmaPlot.
Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)

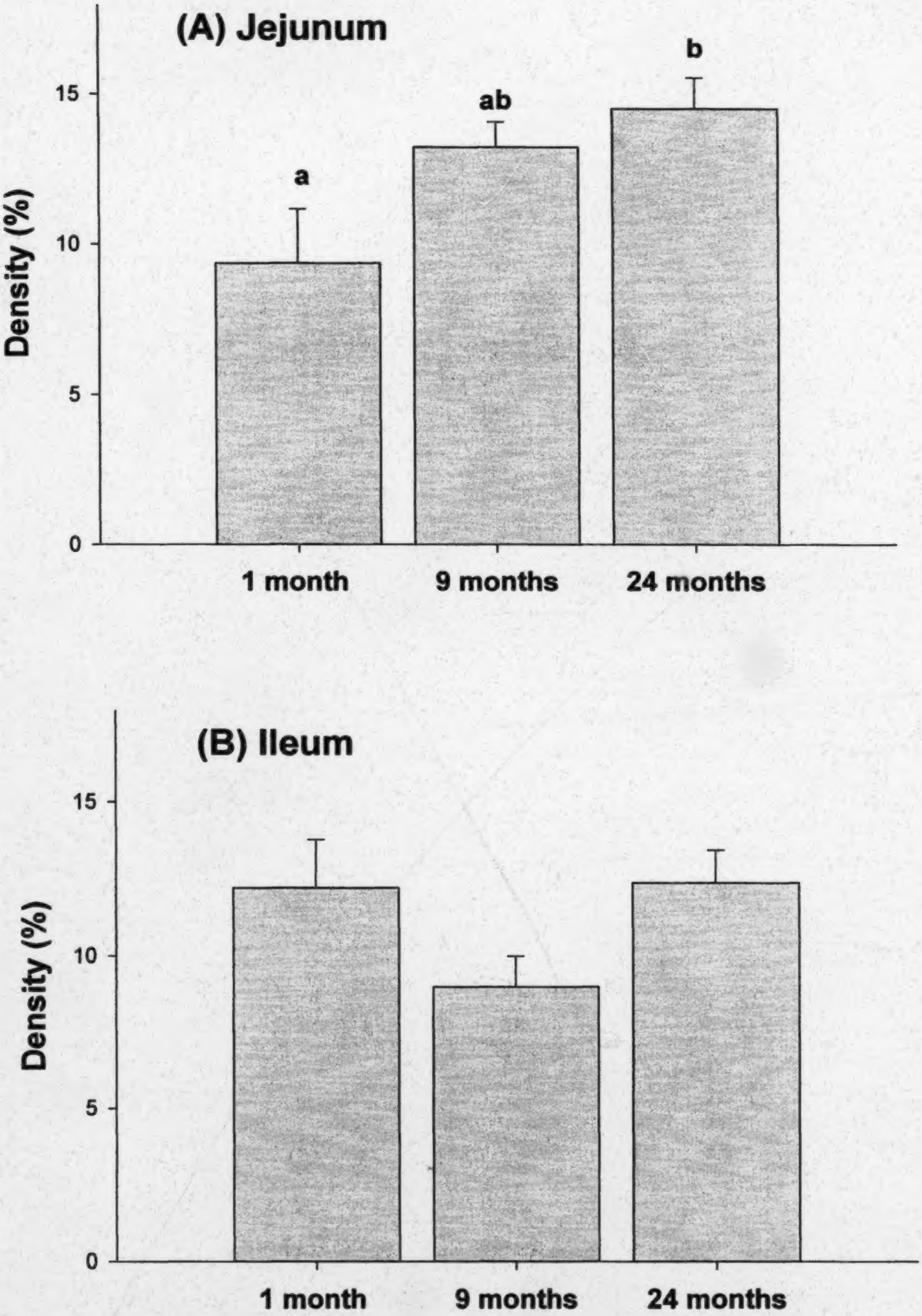
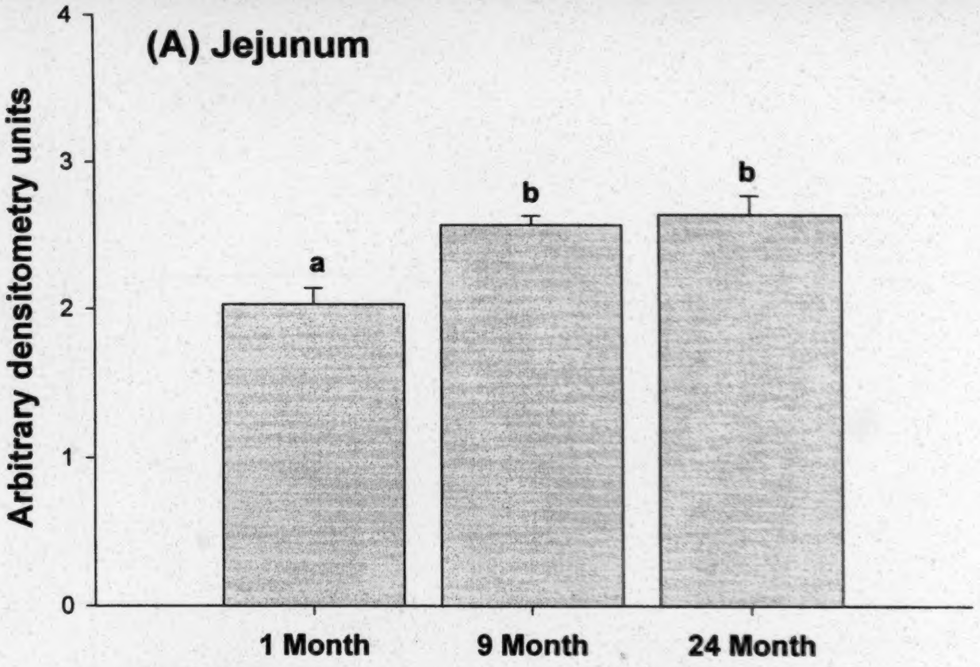


Figure 27. Effect of age on GLUT5 protein abundance as determined by Western blotting in F344 rats.
Values are mean ± SEM
Different letters denote a significant age effect ($p<0.05$)



(A) Jejunum

(B) Ileum

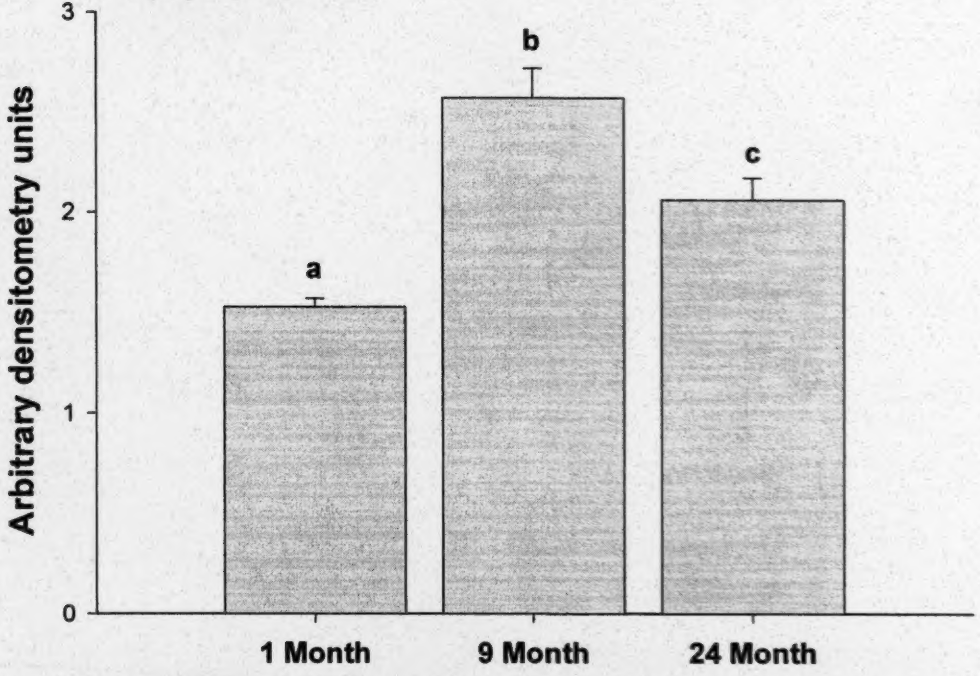
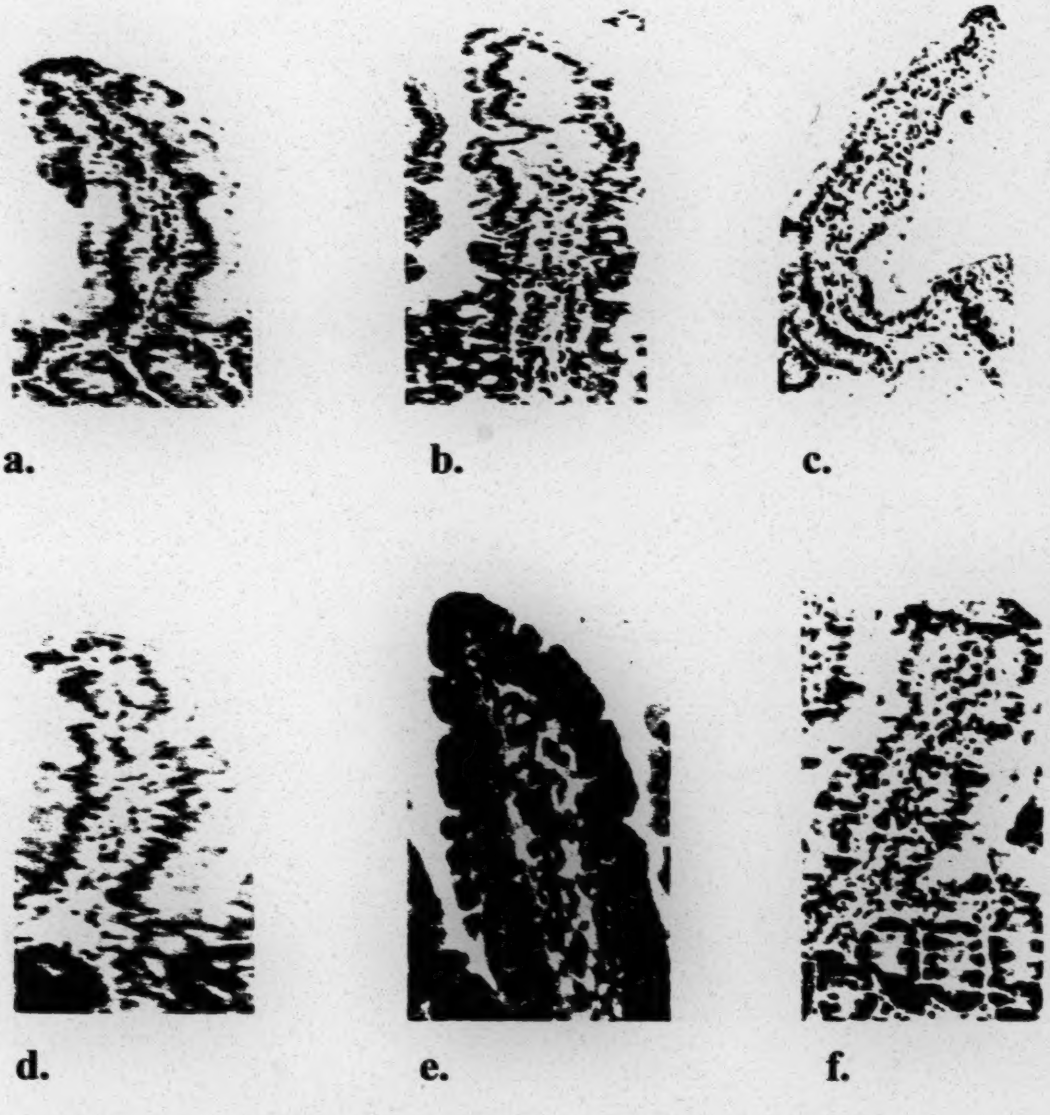


Figure 28. Effect of age on GLUT5 protein abundance as determine by immunohistochemistry in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$).



a. b. c.

d. e. f.

Figure 29. GLUT5 immunohistochemistry on jejunal sections from a) 1 month old rats; b) 9 month old rats; c) 24 month old rats; and ileal sections from d) 1 month old rats; e) 9 month old rats; f) 24 month old rats.

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6. FEEDING A POLYUNSATURATED FATTY ACID DIET PREVENTS THE AGE-ASSOCIATED DECLINE IN GLUCOSE UPTAKE OBSERVED IN RATS FED A SATURATED FATTY ACID DIET

6.1. Introduction

The aging of the population has focused attention on the physiological processes associated with aging, as well as strategies to improve the quality of life for the elderly. The elderly are at a high risk for malnutrition. While there are many physiological and social factors involved, a reduction in nutrient absorption may contribute to the malnourishment. A study using breath hydrogen analysis following a carbohydrate meal showed evidence of malabsorption in the elderly (Feibusch and Holt, 1982). Similarly, transport experiments using isolated brush border membrane (BBM) vesicles demonstrated a reduction in Na+-dependent D-glucose uptake in older patients (Vincenzini et al., 1989). In contrast, a study by Wallis and co-workers (1993) did not find changes in Na+-dependent glucose transport in BBM vesicles isolated from duodenal biopsies from elderly patients.

Experiments using rodent models of aging also demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985). Depending upon the intestinal site, a normal or increased absorptive capacity along the length of the small intestine was found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption: Ferraris and colleagues showed a reduction in uptake and site density of SGLT1 in aged mice (Ferraris et al., 1993; Ferraris and Vinnekota, 1993). This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice.

The discrepancies in the results from human, rat and mouse studies may be due to the differences in the methodologies that were used. While some investigators study uptake using BBM vesicles (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985; Vincenzini et al., 1989; Wallsi et al., 1993), others used everted intestinal rings (Darmenton et al., 1989; Ferraris and Vinnekota, 1993;

Thompson et al., 1988). As well, the method of expressing results is also important. Most studies have expressed uptake based on intestinal weight, and have therefore failed to take into account any potential age- or diet- associated changes in mucosal weight or intestinal surface area. The strain of the animals used, the ages of the animals, and the site of the intestine used may differ between studies, and may explain the variability in the results.

The sodium-dependent glucose transporter in the BBM, SGLT1, is responsible for the transport of glucose and galactose into the enterocyte (Wright et al., 1994). The sodium gradient required for SGLT1 activity is maintained by the Na^+K^+-ATPase in the basolateral membrane (BLM) (Horisberger et al., 1991). The uptake of fructose across the BBM is mediated by GLUT5, a sodium-independent facilitative transporter (Thorens, 1996). The transport of glucose, galactose and fructose out of the enterocyte across the basolateral membrane (BLM) occurs via the facilitative sodium-independent GLUT2 transporter (Thorens, 1996). In addition to its role as a BLM transporter, GLUT2 has recently been localized in the BBM, where it has been suggested to contribute to the uptake of sugars into the enterocyte (Helliwell et al., 2000a; Helliwell et al., 2000b; Kellett, 2001; Kellett and Helliwell, 2000).

Intestinal adaptation is a process by which the intestine changes morphologically and/or functionally in response to alterations in environmental stimuli. Dietary manipulations have been shown to modify intestinal adaptation and nutrient absorption. For example, young animals fed a diet enriched in saturated fatty acids (SFA) have greater glucose uptake than do animals fed an isocaloric diet high in polyunsaturated lipids (PUFA) (Thomson et al., 1986; Thomson et al., 1987; Thomson et al., 1988).

The intestinal adaptive process may be compromised during the process of aging and may thereby contribute to malabsorption and malnutrition. Ferraris and Vinnekota (1995) showed in aging mice that the adaptive increases in uptake in response to a high carbohydrate diet were reduced in old as compared with young animals, and that the changes were limited to the more proximal regions of the intestine. In other studies, following a 3 day starvation period, in rats refed for 1 day, the specific activities of lactase, sucrase and maltase demonstrated an exaggerated enzyme response in aged animals (Holt and Kotler, 1987; Holt et al., 1988).

It is not known how the aging rat responds to diets high in PUFA or SFA, or if modifications in dietary lipids could be used to alter the expected age-associated changes in nutrient absorption. Accordingly, the objectives of this study were to determine (1) the effects of aging on the *in vitro* uptake of glucose in rats; (2) the effect of feeding SFA versus PUFA; and (3) the molecular mechanisms of these age- and diet-associated changes.

6.2. Materials and Methods

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and the Council of the American Physiological Society, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Alberta. Thirty six male Fischer 344 rats, aged 1, 9 and 24 months were obtained from the National Institute of Aging colony and Harlan Laboratories, Maryland, D.C. Pairs of rats were housed at a temperature of 21°C, with 12 h of light and 12 h of darkness. Water and food were supplied *ad libitum*.

Animals were fed standard Purina® rat chow for one week, then randomly assigned to receive one of two diets for a further two weeks: a semi-purified diet containing 20% (w/w) fat and enriched with either SFA or the same diet enriched with PUFA (30) (Tables 9 and 10). There were a total of 6 animals in each age and diet combination. The isocaloric semi-purified diets were nutritionally adequate, providing for all known essential nutrient requirements. Animal weights were recorded at weekly intervals.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labelled probes included D-glucose and L-glucose. The labelled and unlabelled probes were supplied by New England Nuclear and Sigma Co. (St Louis, MO) respectively. The concentrations used were: D-glucose, 2, 4, 8, 16, 32 and 64 mM; and L-glucose, 16 mM. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume.

Tissue preparation

The animals were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbitol, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with cold saline. The intestine was opened along its mesenteric border, and pieces of the proximal segment (jejunum) and distal segment (ileum) were cut and mounted as flat sheets in the transport chambers. A 5 cm piece of each jejunal and ileal segment was gently scraped with a glass slide to determine the percentage of the intestinal wall comprised of mucosa. The chambers were placed in preincubation beakers containing oxygenated Krebs-bicarbonate buffer (pH 7.2) at 37 °C, and tissue discs were preincubated for 15 min to allow the tissue to equilibrate at this temperature. The rate of uptake of glucose was determined from the timed transfer of the transport chambers to the incubation beakers containing [^{3}H]-inulin and ^{14}C-labelled glucose in oxygenated Krebs-bicarbonate (pH 7.2, 37°C). Preincubation and incubation chambers were mixed with circular magnetic bars at identical stirring rates, which were precisely adjusted using a strobe light. Stirring rates were reported as revolutions per minute (rpm). A stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer (Lukie et al., 1974; Westergaard and Dietschy, 1974; Westergaard and Dietschy, 1976).

Determination of uptake rates

After incubating the discs in labelled solutions for 6 min, the experiment was terminated by removing the chamber and rinsing the tissue in cold saline for approximately 5 s. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, placed on a glass slide, and dried overnight in an oven at 55°C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Lukie et al., 1974).

The rates of uptake of glucose were determined as nmol 100 mg tissue $^{-1}$ min $^{-1}$, nmol 100 mg mucosal tissue $^{-1}$ min $^{-1}$, nmol cm $^{-2}$ serosal surface area min $^{-1}$, and nmol cm $^{-2}$ mucosal surface area min $^{-1}$. The value of the kinetic constants was determined using SigmaPlot 2000 (SPSS) (Table 12), and the reproducibility of values was confirmed using Enzfitter (Version 1.05, Elsevier-Biosoft). Statistical significance was accepted for values with $p<0.05$.

Morphology, messenger RNA and protein analysis

Tissue preparation

An additional 24 animals (4 in each of the 6 age/diet groups) were raised and sacrificed similarly as for the uptake studies. A 5 cm portion from each of the proximal jejunum and distal ileum was rinsed, quickly harvested, snap frozen in liquid nitrogen, and stored at –80°C for subsequent mRNA isolation. Mucosal scrapings were harvested from the remaining proximal and distal small intestine, snap-frozen in liquid nitrogen, and stored at –80°C for later isolation of cellular components. For morphological and immunohistochemical analysis, two 1 cm pieces of proximal and distal small intestine were fixed in 10 % formalin.

Morphological Measurements

Morphometric data was obtained from hematoxylin and eosin stained paraffin sections. Measurements were taken of villous height, villous width at one-half villous height, villous bottom width, and crypt depth. Horizontal cross sections were prepared so that villous thickness could be measured at one-half villous height. Magnification was calibrated using a micrometer. Mucosal surface area was calculated as described previously (Thomson et al., 1986). The number of villi per millimeter of serosal length was measured in longitudinal and horizontal cross sections, and multiplied together to obtain the number of villi per square millimeter serosa. When this villous density was multiplied by villous surface area, the result was the mucosal surface area, expressed as square millimeters per square millimeter of serosa. At least 10 villi were assessed per section. The following two formulae were used (Thomson et al., 1986):

Villous surface area (μm^2/villus)= (2 x M x H) + (2 x M–A) x D + (2 x D) x $[(A-M)^2 + (H)^2]^{0.5}$ x 1000, where H=villous height, M=villous width at one-half height, A=villous bottom width, and D=villous thickness.

Mucosal surface area (mm^2/mm^2 serosa) = number of villi/mm^2 serosa x villus surface area (μm^2/villus)/1000

Messenger RNA abundance

The intestinal pieces were homogenized in a denaturing solution containing guanidinium thiocyanate, using The Fast Prep® cell disruptor (Savant Instruments Inc., Holbrook, New York.) Following addition of 2 M sodium acetate, a phenol chloroform extraction was performed. The upper aqueous phase containing the RNA was collected and RNA was precipitated with isopropanol, and washed with 70% ethanol. The concentration and purity of RNA was determined by spectrophotometry at 260 and 280 nm. Samples were stored at -80°C until use for Northern blotting or for reverse transcription polymerase chain reaction (RT-PCR).

Fifteen (15) ug of total RNA was fractionated by agarose gel electrophoresis and transferred to nylon membranes by capillary diffusion. RNA was fixed to the membrane by baking at 80°C for 2 hr. Northern blotting was performed used the DIG Easy Hyb ® method, according to the manufacturers protocol (Roche Diagnostics, Quebec, Canada). The α1 and β1 Na^+K^+-ATPase plasmids were generous gifts from Dr J. Lingrel, University of Cincinnati. The SGLT1 plasmid was kindly donated by Dr Davidson, University of Chicago, and the GLUT2 plasmid was a gift from Dr G.I. Bell, of Howard Hughes Medical Institute, University of Chicago.

The density of the mRNA bands was determined by transmittance densitometry (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario). Quantification of the 28 S ribosomal units from the membranes was used to account for loading discrepancies.

Protein analysis

Brush border membranes (BBM), basolateral membranes (BLM), and enterocyte cytosol were isolated from rat intestinal mucosal scrapings using by differential centrifugation, and Ca^{2+} precipitation (21,23,24). Aliquots were stored at -80°C.

The protein concentration of the samples was determined using the Bio-Rad Protein Assay® (Life Science Group, Richmond, CA). Proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and were transferred to nitrocellulose membranes. Transfer efficiency was verified by Ponceau S staining of membranes, and by Coomassie Blue staining of gels.

Membranes were blocked by incubation overnight in BLOTTO (Bovine Lacto Transfer Technique Optimizer) containing 5% w/v dry milk in Tween Tris Buffered Saline (TTBS) (0.5% Tween 20, 30mM Tris, 150 mM NaCl). Membranes were washed in TTBS, and were subsequently probed with specific rabbit anti-rat antibodies to SGLT1 (Chemicon International, Temecula, California), GLUT2 (Biogenesis, Poole, England) or to $\alpha 1$ Na^+K^+-ATPase and $\beta 1$ Na^+K^+-ATPase (Upstate Biotechnology, Lake Placid, NY). Membranes were incubated for 2 hours at room temperature, with antibodies to GLUT2, $\beta 1$ Na^+K^+-ATPase and $\alpha 1$ Na^+K^+-ATPase. Membranes were incubated overnight at room temperature with SGLT1 antibody. Antibodies were diluted in 2% dry milk in TTBS at 1:500 (SGLT1, GLUT2 and $\alpha 1$ Na^+K^+-ATPase) and 1:1000 ($\beta 1$ Na^+K^+-ATPase). Membranes were subsequently washed with TTBS to remove the residual unbound primary antibody, and were then incubated for 1 hr with goat anti-rabbit antibody (1:20000 in 2 % dry milk in TTBS) conjugated with horseradish peroxidase (HRP) (Pierce, Rockfort, Illinois, USA).

Membranes were washed again in TTBS to remove residual secondary antibody, and were briefly incubated with Supersignal® Chemiluminescent-HRP

Substrate (Pierce, Rockfort, Illinois, USA). Membranes were exposed to X-OMAT AR films, and the relative band densities were determined by transmittance densitometry using Bio-Rad Imaging Densitometer (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario).

Immunohistochemistry

Jejunal and ileal tissue was embedded in paraffin and 4-5 micron sections were mounted on glass slides. The sections were dewaxed in xylene, and hydrated after incubation with a series of ethanol treatments. Slides were then incubated in hydrogen peroxide/methanol solution (20% - 50% H_2O_2, 80% methanol) for 6 min, rinsed in tap water, and counterstained with Harris Hematoxylin. Slides were then air-dried, and the tissue was encircled with hydrophobic slide marker (PAP pen, BioGenex, California). After rehydration in phosphate buffered saline (PBS), the slides were incubated for 15 min in blocking reagent (20% normal goat serum), followed by primary antibody to SGLT1 for 30 min. Slides were then washed in PBS and incubated in LINK® and LABEL® according to the manufacturers protocol. The slides were subsequently incubated for 5 min in DAB®, rinsed in water, dehydrated in absolute ethanol, and cleared in xylene. Negative controls were processed on the same slide in an identical manner, excluding the incubation with the primary antibody. A Leitz Orthoplan Universal Largefield microscope and a Leitz Vario Orthomat 2 automatic microscope camera were used to photograph the slides. Chromagen staining was quantified using a Pharmacia LKB-Imagemaster DTS densitometer and Pharmacia Imagemaster 1D (Version 1.0) software (Amersham Pharmacia Biotechnology Inc, Piscataway, New Jersey, USA). Villi were divided into 5 equal sections from the villous tip to the crypt. Protein abundance was quantified in each section, and compared in order to determine the distribution of protein along the crypt-villous axis. Four villi per animal were quantified, and the results were normalized to the negative control values.

Expression of results

The results were expressed as mean ± standard error of the mean. The statistical significance of the differences between the three age groups was determined by analysis of variance (ANOVA) ($p<0.05$). Individual differences between ages were determined using a Student-Neuman-Keuls multiple range test. The statistical significance for diet effect (SFA versus PUFA) was determined using Student's t-test ($p<0.05$).

6.3. Results

Animal Characteristics

The rate of body weight change (grams per day) fell between 1, 9 and 24 months in rats fed SFA or PUFA (Figure 30). At each age, the body weight was lower with SFA than with PUFA. Food intake was not influenced by the age of the rats, regardless of whether they were fed SFA or PUFA (data not shown). Age and diet had no effect on the weight of the jejunum, the weight of the scraped jejunal mucosa, or on the percentage of the jejunal wall comprised of mucosa (Table 11). Similarly, in the ileum, age and diet had no effect on the mucosal weight or on the percentage of the intestinal wall comprised of mucosa. In the ileum of rats fed SFA, tissue weight at 1 month was lower than in those fed PUFA, and ileal weight was lower at 24 than at 9 months. In those fed PUFA, ileal weight was lower at 9 and 24 months than at 1 month. Therefore, the weight of the mucosa had to be taken into account when expressing the rate of uptake of glucose.

There were no differences in the mean values of the heights of the villi of the jejunum or ileum of rats aged 1, 9 or 24 months (data not shown). In animals fed SFA, the jejunal and ileal mucosal surface areas were lower at 9 and 24 months, as compared with 1 month (Figure 31). In those fed PUFA, both the jejunal and ileal mucosal surface area were lower at 24 as compared with 1 and 9 months. In the jejunum, diet had no effect on mucosal surface area at 1, 9 or 24 months (Figure 31). In the ileum of animals fed PUFA as compared to SFA, mucosal surface area was higher at 9 months but lower at 24 months. While there were no significant differences in jejunal mucosal surface area at 1, 9 or 24 months in animals fed SFA as compared with PUFA, the age-associated decline in jejunal or mucosal surface area occurred between 9 and 24 months in rats fed PUFA, and between 1 and 9 months in those fed SFA. Thus, feeding PUFA slowed the age-associated decline in the mucosal surface area of the jejunum and ileum. The surface area of the mucosa, therefore, had to be taken into account when expressing the rate of glucose uptake.

Glucose Uptake

When the rates of uptake of 64 mM glucose were expressed on the basis of the weight of the full wall of the intestine (nmol 100 mg^{-1} min^{-1}), in animals fed SFA there were no changes in jejunal uptake of glucose with age. However, decreased ileal uptake was found at 9 and 24 months as compared to 1 month (Figure 32). In animals fed PUFA, there were no changes in jejunal or ileal uptake of glucose with age.

When glucose uptake was expressed on the basis of mucosal weight (nmol 100 mg mucosa^{-1} min$^{-1)}$, in rats fed SFA there was no change in jejunal uptake of glucose between 1 and 24 months, but the ileal uptake of glucose fell between 1 and 9 and between 1 and 24 months (Figure 33). In rats fed PUFA, there was no change in jejunal or ileal uptake of glucose between 1 and 24 months.

When glucose uptake was expressed on the basis of serosal surface area, age and diet had no influence on glucose uptake (data not shown). When the rate of glucose uptake was expressed on the basis of mucosal surface area, in rats fed SFA the jejunal uptake of glucose was higher at 9 and 24 months as compared to 1 month old animals (Figure 34). In the ileum of rats fed SFA, uptake was increased at 9 months but not at 24 months, as compared to 1 month. In animals fed PUFA, jejunal and ileal uptake of glucose was higher at 24 than at 9 or 1 month.

The passive component of D-glucose uptake was estimated from the rate of uptake of L-glucose. Age and diet had no influence on the jejunal or ileal uptake of L-glucose (data not shown).

Transporter Protein Abundance

Using Western blotting, the abundance of SGLT1 in the BBM of the jejunum was similar at 1, 9 and 24 months in rats fed SFA or PUFA (Figure 35). In rats fed PUFA, SGLT1 abundance in the ileum was similar at 1, 9 and 24 months. In rats fed

SFA, SGLT1 abundance in the ileum was lower at 24 as compared with 1 or 9 months.

The jejunal abundance of SGLT1 determined by immunohistochemistry was decreased between 24 and 1 month in rats fed SFA or PUFA (Figure 36). In PUFA, a reduction in the ileal abundance of SGLT1 was seen between 24 and 1 month. SFA fed rats showed an increase in ileal abundance of SGLT1 at 9 months, when compared to 1 or 24 month old animals. One month old rats fed SFA showed a reduction in SGLT1 abundance when compared to animals fed PUFA. Jejunal and ileal villi were divided into 5 equal sections starting from the tip of the villi down to the crypt region. The abundance of SGLT1 protein was evenly distributed along the crypt-villous axis (Figure 39) and SGLT1 distribution was not affected by age or dietary treatment (data not shown).

In animals fed SFA, the jejunal BLM abundances of α1 Na^+K^+ATPase and β1 Na^+K^+ ATPase were lower at 24 months as compared with 9 or 1 month, but was unchanged in the ileum (Figure 37 and 38). In animals fed PUFA, the jejunal α1 Na^+K^+ATPase and β1 Na^+K^+ATPase abundances were similar at 1, 9 and 24 months. In the ileum of rats fed PUFA, α1 Na^+K^+ATPase protein abundance was reduced at 24 months as compared to 9 months. The abundance of GLUT2 in the BML was similar at 1, 9 and 24 months in animals fed SFA or PUFA (data not shown)

Transporter mRNA Abundance

In animals fed SFA or PUFA, the jejunal and ileal SGLT1 mRNA abundance was unaffected by age (data not shown). The jejunal and ileal α1 Na^+K^+ATPase and β1 Na^+K^+ATPase mRNA abundances were similar at 1, 9 and 24 months of age in animals fed SFA or PUFA (data not shown). GLUT2 mRNA abundance was similar at 1, 9 and 24 months in animals fed SFA or PUFA (data not shown).

6.4. Discussion

Variations in dietary lipids influence the changes in intestinal surface area seen with aging. Animals fed SFA exhibited a reduction in jejunal and ileal surface area at both 9 and 24 months (Figure 31). This decrease in jejunal surface area seen with aging is delayed by feeding PUFA, as animals fed this diet showed reductions in surface area only at 24 months of age. In addition, feeding PUFA prevents the age-associated decline in ileal mucosal surface area seen with SFA. This signifies that the intestine of the old rats retains its ability to exhibit adaptive changes in its morphology in response to dietary lipid manipulations. This observation also stresses the point that failure to take into account the animal's diet may lead to errors in the interpretation of the effects of aging on the morphology of the intestine. Finally, the rate of decline in jejunal mucosal surface area seen in rats fed SFA can be slowed by feeding PUFA, and the age-associated decline in ileal mucosal surface area seen in SFA is prevented by feeding PUFA.

The simplest way of expressing the rate of *in vitro* uptake of nutrients is on the basis of the weight of the full thickness of the wall of the intestine. However, if a treatment alters the weight of the intestine, then there may be variations in the rate of nutrient uptake which are understandable in the light of there simply being more mucosal tissue or a greater surface area. For this reason, where there are treatment-associated variations in mucosal mass or the surface area of the villous membrane, as was the case in this study (Table 11 and Figure 31), then it is also appropriate to express solute uptake on the basis of the mass of the transporting mucosal tissue or the mucosal surface area. Clearly, aging reduces ileal glucose uptake when expressed on the basis of intestinal or mucosal weight (Figures 32 and 33), but increases jejunal and ileal uptake when changes in mucosal surface area are taken into account (Figure 34). Thus, the ability to show an effect of age or diet on glucose uptake depends on the way in which the rate of uptake is expressed.

Previous studies have shown that sugar uptake is increased in young rats fed SFA as compared to PUFA (Thomson et al., 1986; Thomson et al., 1987; Thomson et al., 1988). The results of this study confirm these findings in 9 month old animals (Figure 34). However, in the ileum of 24 month old animals the relationship is reversed, as glucose uptake is increased with PUFA as compared to SFA (Figure 34). This suggests that dietary lipids influence the absorption of nutrients differently depending upon the age of the animal. This finding is in agreement with other work

(Ferraris and Vinnekota, 1995; Holt and Kotler, 1987; Holt et al., 1988) that shows a reduction in the adaptive capabilities of the intestine in response to diet with aging. Ferraris and Vinnekota (1995) noted that the effect of dietary changes in aged mice was limited to the proximal small intestine, but the effect of dietary lipids on glucose uptake in aged rats occurs in both the jejunum and ileum (Figures 32-34). Thus, aging modifies the adaptability of the intestine in response to dietary manipulations. Clearly, the influence of a given manipulation that results in intestinal adaptation in young rats does not necessarily apply to older animals.

Despite the decreases in surface area of the intestine seen with aging (Figure 31), jejunal glucose uptake, when expressed on the basis of mucosal surface area, is increased with age in animals fed PUFA or SFA (Figure 34). Importantly, the age-associated decline in the ileal uptake of glucose observed in rats fed SFA, can be prevented by feeding PUFA (Figures 32 and 33). This may have contributed to the lower rate of weight loss in rats fed PUFA as compared with those fed SFA (Figure 30). With both SFA and PUFA, the jejunal SGLT1 abundance shown by Western blotting did not decline with aging (Figure 35), and yet did decline when examined by immunohistochemistry (Figure 36). Despite this, with aging the jejunal uptake of glucose was either unchanged or rose (Figures 32-34). The ileal abundance of SGLT1 fell with SFA but not PUFA when assessed by Western blotting (Figure 35), yet fell with PUFA but not SFA when assessed by immunohistochemistry (Figure 36). In SFA the lower ileal SGLT1 on Western blotting, was associated with a lower rate of uptake of glucose (Figures 32 and 33), whereas the lower ileal SGLT1 on immunohistochemistry in PUFA was associated with either no change (Figures 32 and 33), or an increase in glucose uptake (Figure 34). Furthermore, the abundance of SGLT1 mRNA did not change with aging or diet (data not shown). Thus, there was no consistent parallel between alterations in glucose uptake, expressed by various methods (Figures 32-34), and variations in the abundance of SGLT1 demonstrated with two methods (Figures 35 and 36) or the abundance of SGLT1 mRNA (data not shown).

Post-translational changes may be occurring in SGLT1 that affect the activity of the glucose transporter. Potential sites for the protein kinase A and protein kinase C have been recognized in SGLT1, and binding sites on SGLT1 for glucose are increased by phosphorylation (Ishikawa et al., 1997). The activity of SGLT1 may be increased without a concomitant increase in the amount of protein detected by Western blotting or immunohistochemistry. A gradient of Na^+ across the BBM is required to achieve full activity of SGLT1, and this gradient is achieved by the Na^+K^+ATPase in the BLM (Horisberger et al., 1991). The increase in jejunal uptake of glucose with SFA in old rats (Figure 34) was associated with a decline in the abundance of jejunal α1 and β1 Na^+K^+ATPase (Figures 38 and 39), and the increase in jejunal uptake of glucose in 24 month old animals fed PUFA (Figure 34) was not associated with any alteration in the protein abundance of α1 or β1 Na^+K^+ATPase. Furthermore, there were no age- and diet-associated changes in Na^+K^+ATPase mRNA abundance (data not shown). Thus, the age- and diet-associated changes in glucose uptake by BBM SGLT1 could not be explained by variations in the protein or mRNA abundance of α1 or β1 Na^+K^+ATPase.

One could speculate that changes in the fluidity of the BBM as a result of aging might explain the apparent uncoupling of transport to gene expression and protein abundance. There are reductions in the membrane fluidity of the BBM isolated from 117 week old Fischer 344 rats as compared to younger animals (Brasitus et al., 1984). Similarly, the fluorescence polarization technique used by Wahnon et al. (1989) showed reductions in membrane fluidity in 19 month old rats when compared to 1 and 9 month old animals. Indeed, a study done using chickens demonstrated that reductions in membrane fluidity, as a result of changes in membrane lipid content, may be involved in the decrease in D-glucose uptake observed during post-hatching development (Vazquez et al., 1997). With aging, declines in membrane fluidity are associated with increases in uptake (Meddings et al., 1990).

Alterations in the fluidity of the BBM as a result of dietary lipid manipulations might also explain the apparent uncoupling of transport to gene expression and protein abundance. Altering the fatty acid composition of the diet results in changes in the phospholipid content of the BBM of enterocytes (Keelan et al., 1997). It is reasonable to speculate that changing the dietary lipids may have altered BBM fluidity and therefore SGLT1 function in the older animals.

In rodent models of diabetes, a "recruitment" of transporters in the lower part of the villi results in active transport occurring in this area, and a resultant increase in glucose transport (Burant et al., 1994). As most intestinal glucose uptake occurs in the upper third of the villi (Thomson et al., 1994), a redistribution of SGLT1 to this area may explain altered uptake. However, this study did not show changes in the immunohistochemical distribution of SGLT1 with age or dietary manipulations (Figure 37), and thus alterations in the distribution of SGLT1 along the crypt-villus axis could not explain variations in glucose uptake with aging.

The contribution of a passive component of glucose absorption has been recognized. Pappenheimer and Reiss (1987) proposed the theory of paracellular solvent drag as the passive component of glucose absorption. Changes in glucose uptake (Figure 32-34) could not be explained by alterations in passive uptake since the uptake of L-glucose was unaffected by age or diet (data not shown). Recently it has been proposed that GLUT2 is present in the BBM (Helliwell et al., 2000a; Kellett, 2001) and transports glucose into the cell via facilitated diffusion. If these findings can be confirmed, then it is possible that the age- and diet-associated alterations in glucose uptake may have been due to changes in the BBM abundance of GLUT2.

Acknowledgements

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Table 9. Fatty acid composition of the semi-synthetic diets

FATTY ACID (% of total)	Diet enriched with saturated fatty acids (SFA)	Diet enriched with polyunsaturated fatty acids (PUFA)
14:0	1.8	0.1
14:1n9	0.0	0.0
15:0	0.2	0.0
16:0	21.8	5.5
16:1n7	0.0	0.1
18:0	61.1	5.2
18:1n9	2.8	16.3
18:1n7	0.1	0.2
18:2n6	9.6	69.2
18:3n3	0.0	0.8
18:3n6	0.0	0.1
18:4n3	0.0	0.0
19:0	0.2	0.0
20:0	1.5	0.2
20:1n9	0.0	0.3
20:1n7	0.1	0.3
20:2n6	0.1	0.2
20:3n9	0.1	0.2
20:3n6	0.0	0.0
20:4n6	0.0	0.0
20:5n3	0.0	0.3
22:0	0.0	0.4
22:1n9	0.0	0.0
22:1n7	0.3	0.3
22:4n6	0.0	0.1
22:5n6	0.0	0.0
22:5n3	0.1	0.1
24:0	0.1	0.1
22:6n3	0.1	0.0

Table 10. Macronutrient composition of the semi-synthetic diets

INGREDIENTS	CONCENTRATION (g/kg diet)
Fat	200.00
Cornstarch	378.00
Casein	270.50
Non-nutritive fiber	80.00
Vitamin mix*	10.00
Mineral mix#	50.00
L-methionine	2.50
Choline	2.75
Inositol	6.25

- * A.O.A.C. vitamin mix (Teklad Test Diets, Madison, WI) provided the following per kilogram of complete diet: 20 000 IU of vitamin A; 2000 IU of vitamin D; 100 mg of vitamin E; 5 mg of menadione; 5 mg of thiamine-HCl; 8 mg of riboflavin; 40 mg of pyridoxine-HCl; 40 mg of niacin; 40 mg of pantothenic acid; 2000 mg of choline; 100 mg of myoinositol; 100 mg of p-aminobenzoic acid; 0.4 mg of biotin; 2 mg of folic acid; and 30 mg of vitamin B12.
- # Bernhart Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH) was modified to provide 77.5 mg of Mn and 0.06 mg Se per kilogram of complete diet.

Table 11. Effect of Age and Diet on Intestinal Weight

	1 Month		9 Months		24 Months	
	SFA	PUFA	SFA	PUFA	SFA	PUFA
Tissue weight (mg/cm)						
Jejunum	8.8 ± 0.9	10.1 ± 1.4	12.5± 0.5	11.2 ± 0.8	13.6 ± 2.1	10.5 ± 1.1
Ileum	6.8 ± 0.6 *	9.4 ± 0.8	10.4 ± 1.9	7.6 ± 1.1 a	8.5 ± 0.9 b	5.4 ± 1.1 a
Mucosal weight (mg/cm)						
Jejunum	4.6 ± 0.7	5.5 ± 0.9	6.3 ± 0.6	5.9 ± 1.0	7.2 ± 1.5	5.6 ± 0.8
Ileum	3.1 ± 0.5	4.7 ± 0.7	5.6 ± 1.1	3.9 ± 1.0	4.2 ± 0.7	2.9 ± 0.7
% of Mucosa						
Jejunum	51.7 ± 3.7	55.0 ± 6.2	50.6 ± 3.7	51.8 ± 6.2	51.4 ± 3.6	51.2 ± 3.4
Ileum	45.6 ± 5.0	49.9 ± 5.3	51.4 ± 3.6	48.8 ± 5.4	49.3 ± 4.0	42.8 ± 8.9

Mean ± SEM

a	significantly different from 1 month old rats $p<0.05$
b	significantly different from 9 month old rats $p<0.05$
*	significantly different from PUFA

Table 12. Effect of Age on the Values of the Maximal Transport Rate (Vmax) for Glucose Uptake (nmol•100 mg mucosa-1 min-1)

	1 month		9 months		24 months	
	SFA	PUFA	SFA	PUFA	SFA	PUFA
Jejunum						
SigmaPlot	4302±362	7037±3384	50281±106367	5116±3347	3937±1504	2612±181
Lineweaver-Burke	1250±179	1429±89	1111±417	833±167	1667±167 *	3333±0 ab
Eadie-Hofstee	1977±235	1894±290	2044±557	1149±112	2092±295	2560±549
Wolfee	3333±0 *	10000±556	5000±1500	2000±500 a	2500±417	2500±417 a
Ileum						
SigmaPlot	21371±17044	5012±1357	4863±2213	$8.5x10^8\pm$	3879±608	5124±470
Lineweaver-Burke	2000±0 *	3333±417	714±104	769±121	2500±67 *	25000±15000 ab
Eadie-Hofstee	2873±178	2374±198	1128±305 a	1359±245 a	2630±2507	4952±1466
Wolfee	5000±0	3333±0	2500±250 *	10000±2500 a	3333±417	5000±3333

Mean ± SEM

a significantly different from 1 month old animals ($p<0.05$)

b significantly different from 9 month old animals ($p<0.05$)

* significantly different from PUFA ($p<0.05$)

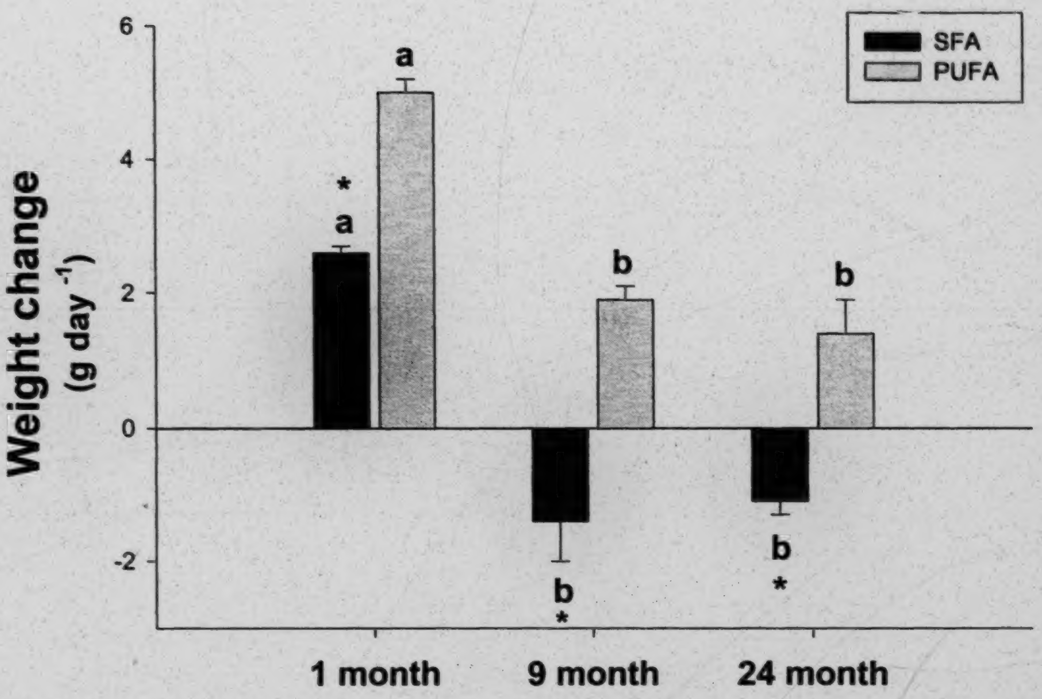


Figure 30. Effect of age and dietary lipids on body weight change in F344 rats. Values are Mean ± SEM.
Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)
* indicates a significant diet effect (SFA vs PUFA) (t-test $p \leq 0.05$)

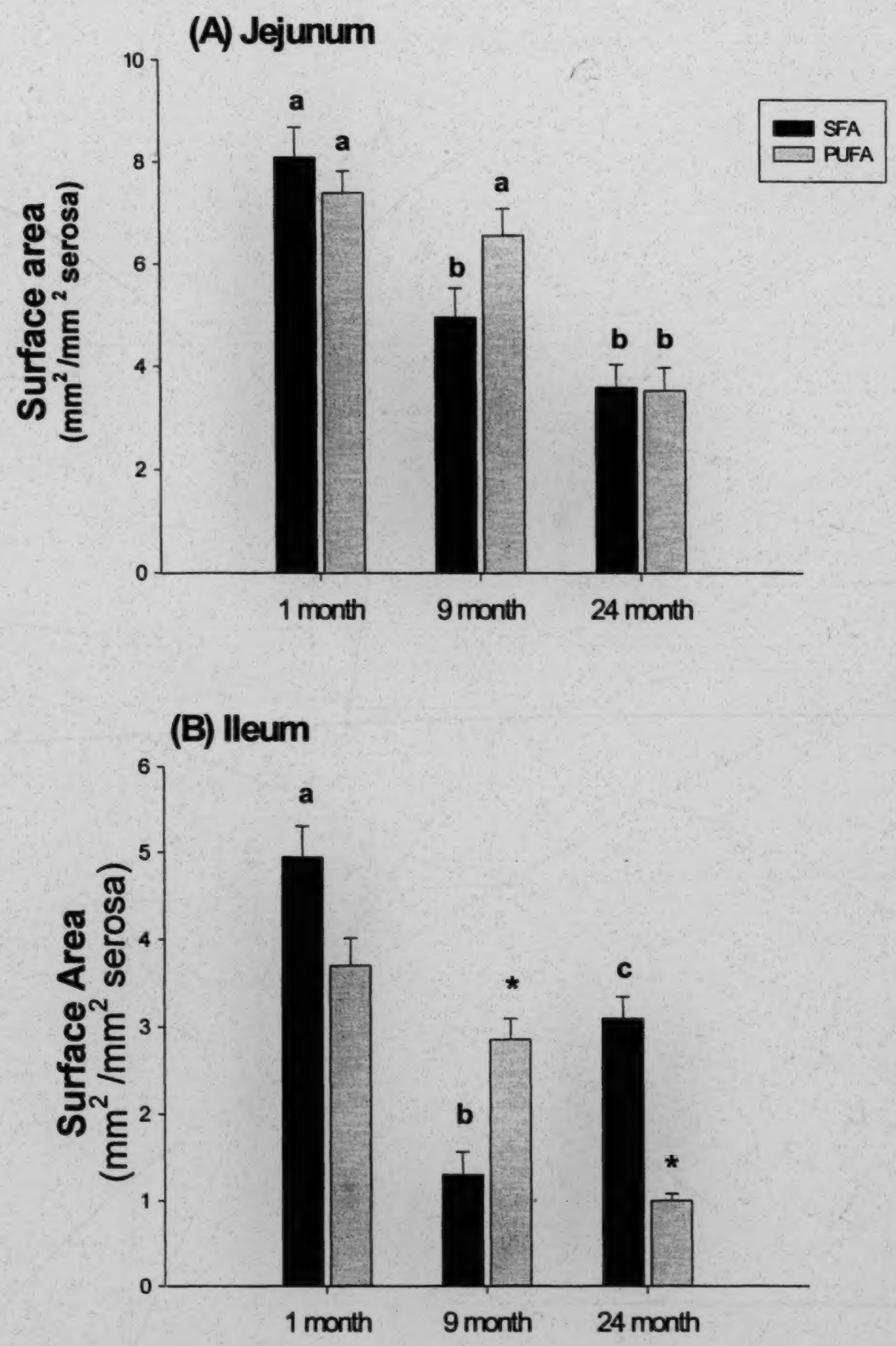
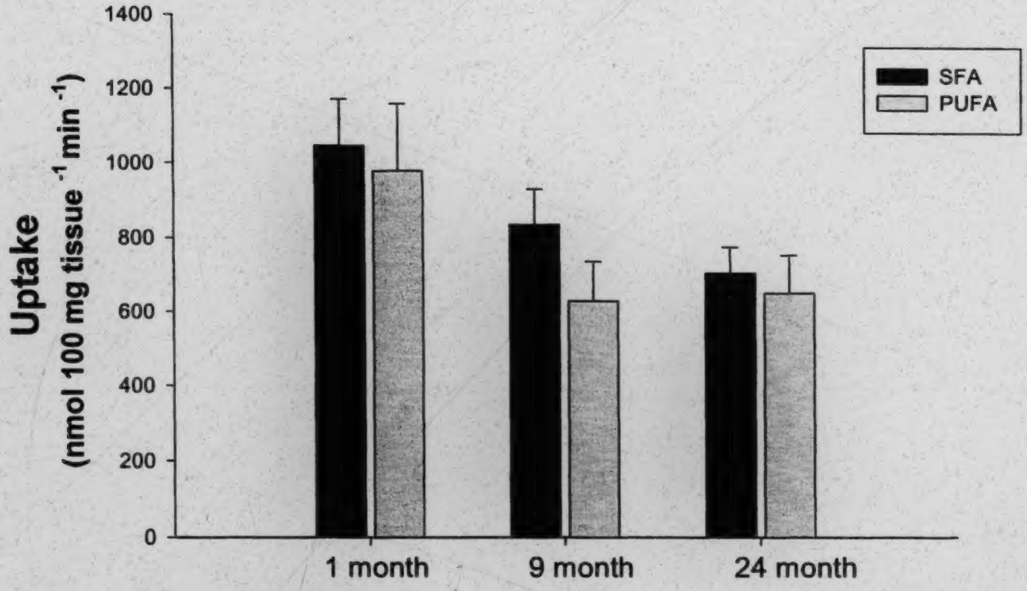


Figure 31. Mucosal surface area of the small intestine in F344 rats
Values are Mean ± SEM.
Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)
* indicates a significant diet effect (SFA vs. PUFA) (t-test $p \leq 0.05$).

(A) Jejunum



(B) Ileum

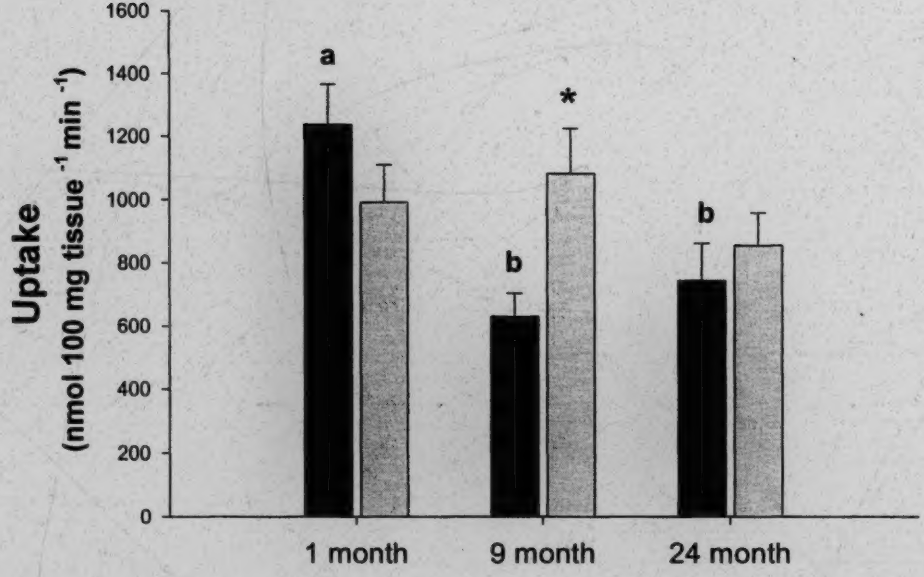
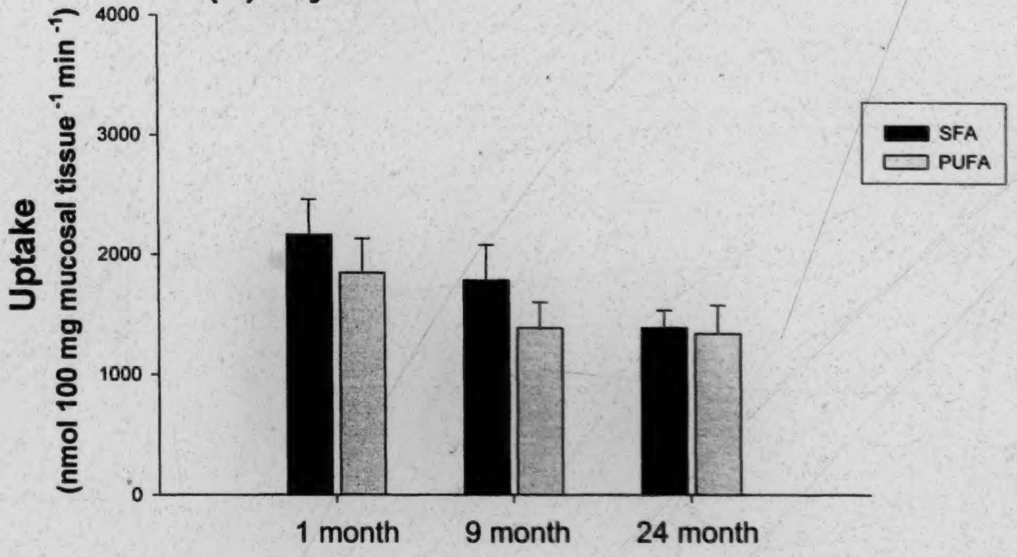


Figure 32. Uptake of 64 mM D-glucose expressed on the basis of intestinal weight in F344 rats. Values are Mean ± SEM
Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)
* indicates a significant diet effect (SFA vs PUFA) (t-test $p \leq 0.05$)

(A) Jejunum



(B) Ileum

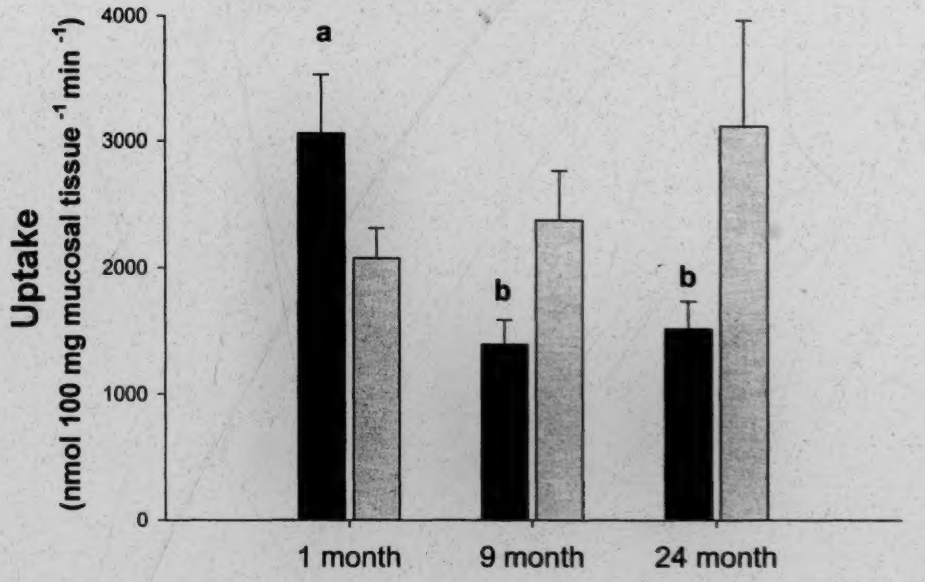


Figure 33. Uptake of D-glucose expressed on the basis of mucosa weight in F344 rats. Values are Mean ± SEM.
Different letters indicate a significant age effect (ANOVA $p \leq 0.05$)
No significant diet effect between (SFA vs PUFA) (t-test $p \leq 0.05$)

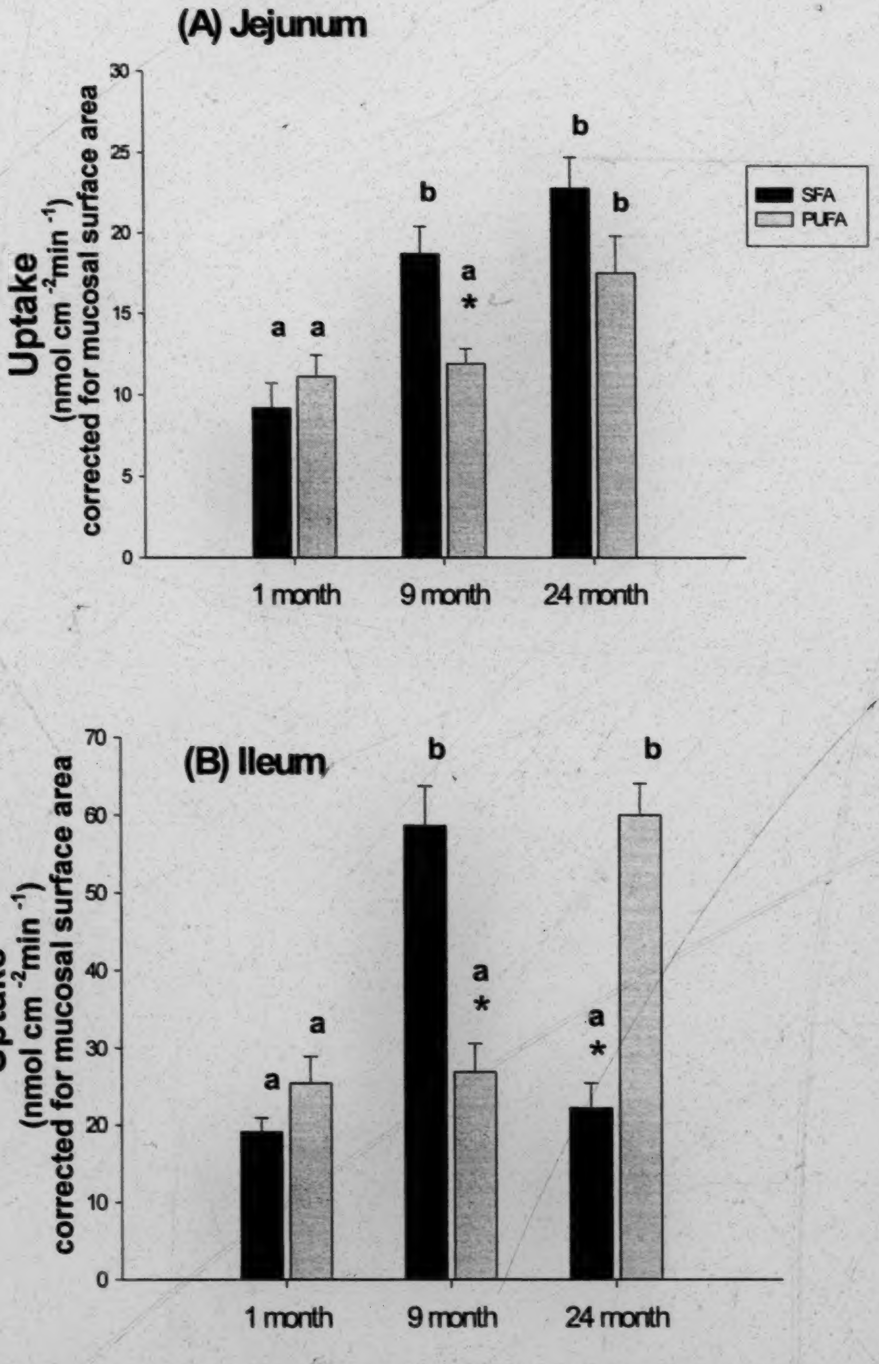
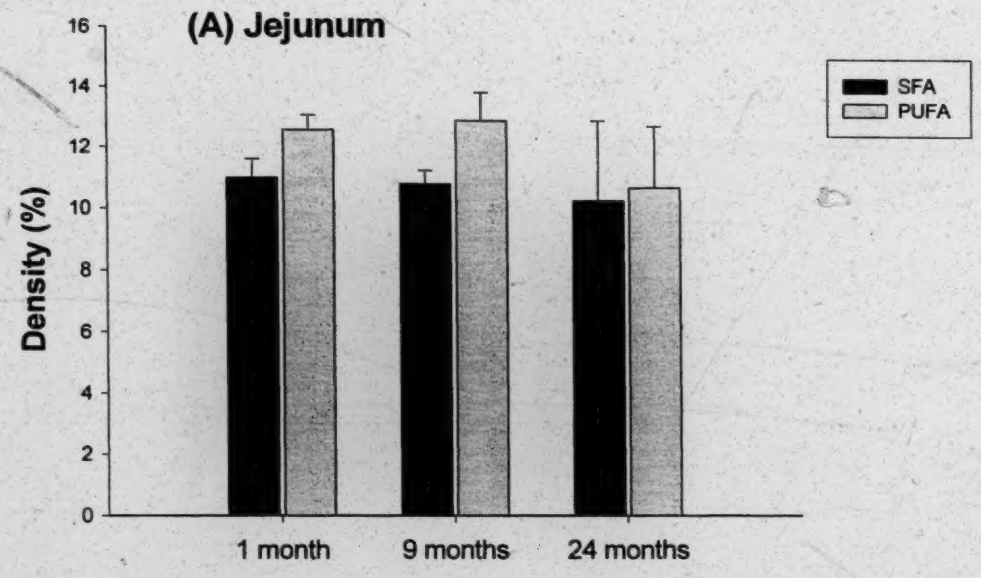


Figure 34. Uptake of D-glucose expressed on the basis of mucosal surface area in F344 rats.Values are Mean ± SEM Different letters indicate a significant age effect (ANOVA $p \leq 0.05$) * indicates a significant diet effect (SFA vs PUFA) (t-test $p \leq 0.05$)



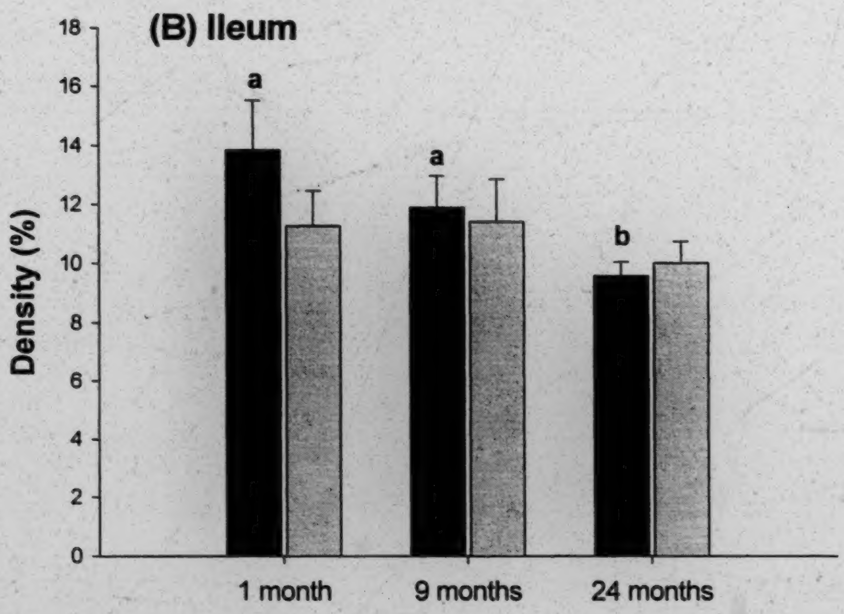


Figure 35. Effect of age and dietary lipids on SGLT1 protein abundance in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$). * denotes a significant diet effect (SFA vs PUFA) ($p< 0.05$).

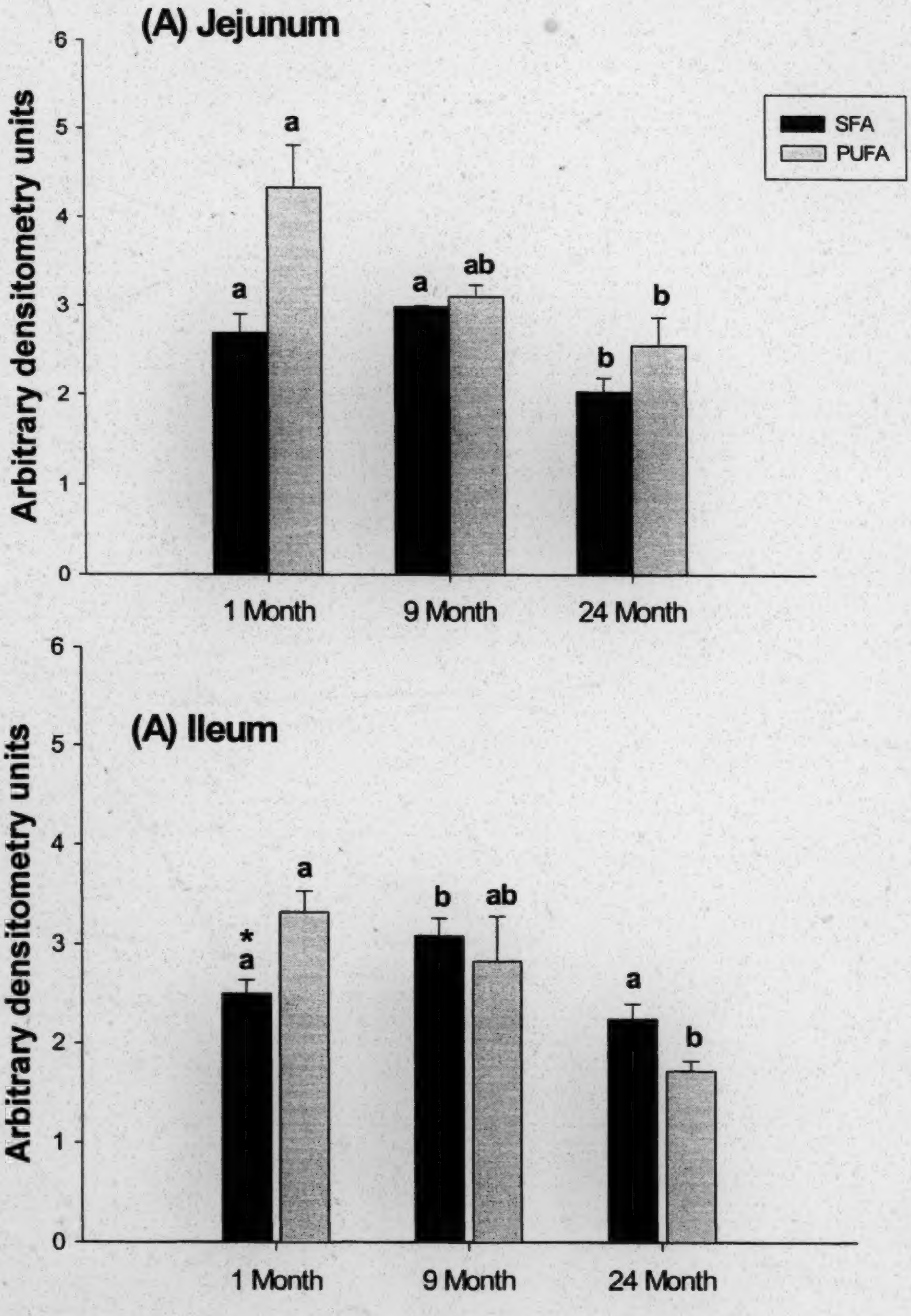
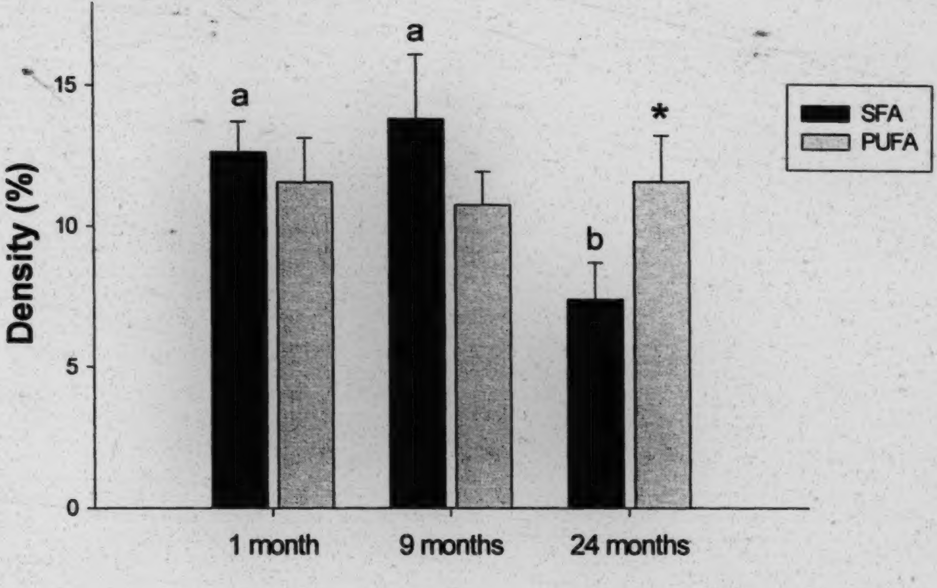


Figure 36. Effect of age and dietary lipids on SGLT1 abundance as determined by immunohistochemistry in F344 rats. Values are mean + SEM. Different letters denote a significant age effect ($p<0.05$). * denotes a significant diet effect (SFA vs PUFA) ($p<0.05$)

(A) Jejunum



(B) Ileum

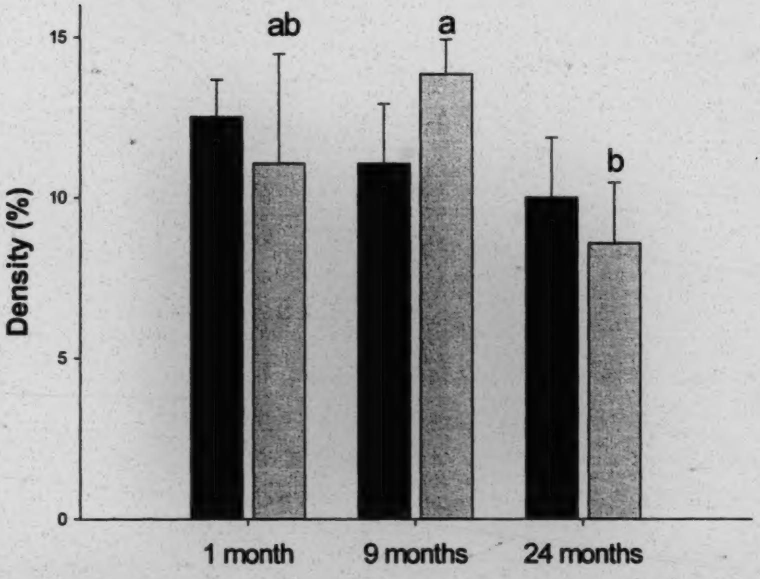


Figure 37. Effect of age and dietary lipids on α1 $Na^{+}K^{+}$-ATPase protein abundance in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$).
* denotes a significant diet effect (SFA vs PUFA) ($p< 0.05$).

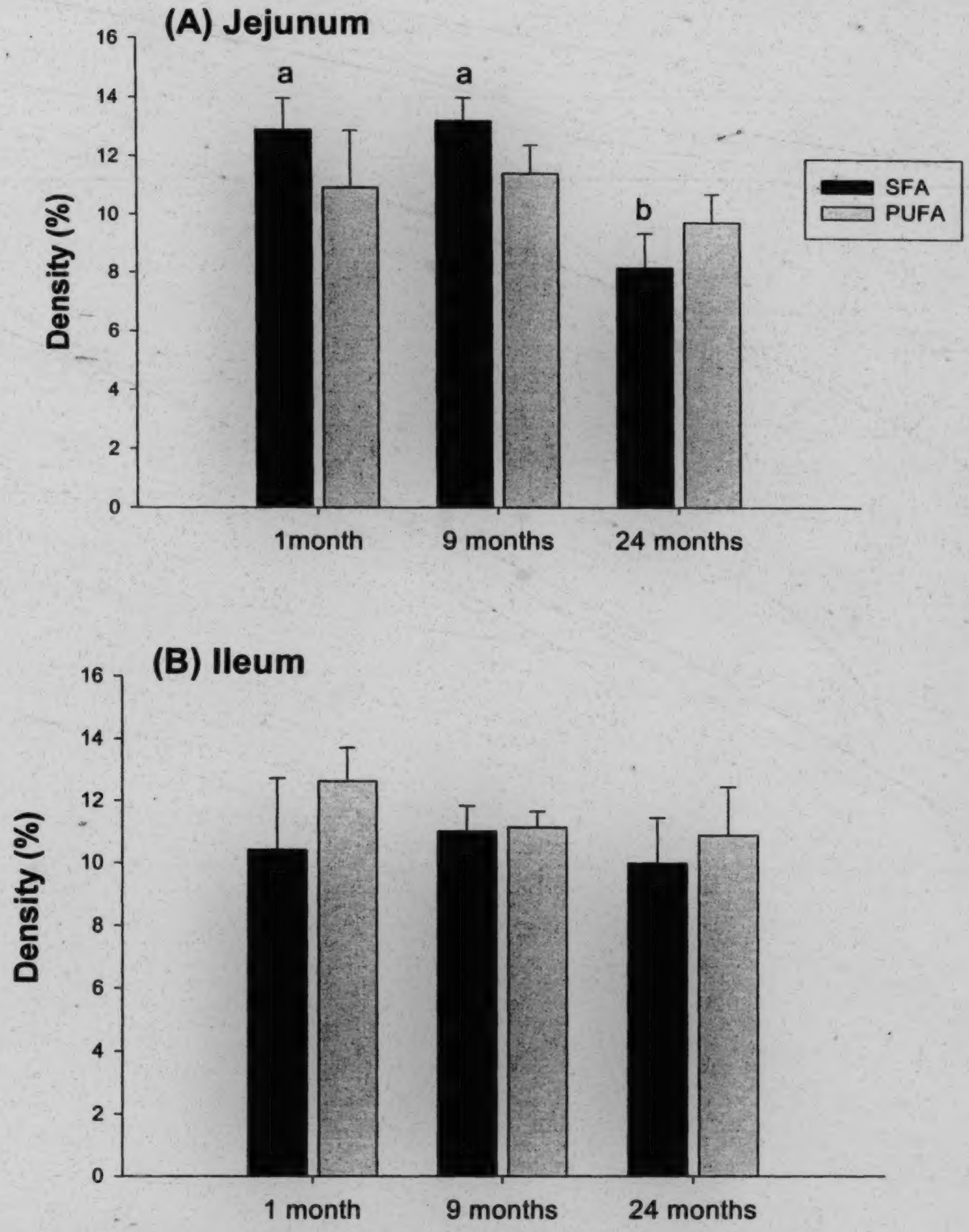


Figure 38. Effect of age and dietary lipids on B1 Na^+K^+-ATPase protein abundance in F344 rats. Values are mean ± SEM. Different letters denote a significant age effect ($p<0.05$) * denotes a significant diet effect (SFA vs PUFA) ($p< 0.05$).

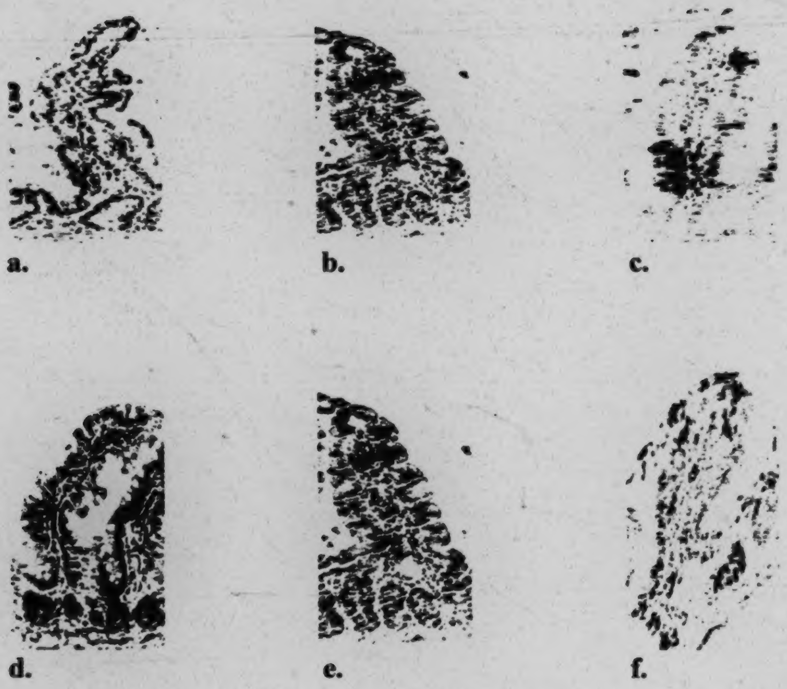


Figure 39. SGLT1 immunohistochemistry on ileal sections from a) 1 month old rats fed PUFA; b) 9 month old rats fed PUFA; c) 24 month old rats fed PUFA; d) 1 month old rats fed SFA; e) 9 month old rats fed SFA; f) 24 month old rats fed SFA

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7. DIETARY LIPIDS MODIFY THE AGE-ASSOCIATED CHANGES IN THE INTESTINAL UPTAKE OF FRUCTOSE IN RATS

7.1. Introduction

The aging of the population has focused attention on the physiological processes associated with aging, as well as strategies to improve the quality of life for the elderly. The elderly are at a high risk for malnutrition. While there are many physiological and social factors involved, a reduction in nutrient absorption may contribute to this malnourishment. A study using breath hydrogen analysis following a carbohydrate meal showed evidence of malabsorption in the elderly (Feibusch and Holt, 1982). Similarly, transport experiments using isolated brush border membrane (BBM) vesicles demonstrated a reduction in Na+-dependent D-glucose uptake in older patients (Vincenzini et al., 1989). In contrast, a study by Wallis and co-workers (1993) did not find changes in Na+-dependent glucose transport in BBM vesicles isolated from human duodenal biopsies.

The results from experiments using rodent models of aging also demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Lindi et al., 1985; Freeman and Quamme, 1986; Doubek and Armbrecht, 1989). However, a normal or increased absorptive capacity along the length of the small intestine was found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice do not offer conclusive results on the effect of aging on nutrient absorption: Ferraris et al. (1993) show a reduction in uptake and site density of SGLT1 in aged mice. This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice.

The discrepancies in the results from human, rat and mouse studies may be due to the differences in the methodologies that were used. While some investigators study uptake using BBM vesicles (Lindi et al., 1985; Freeman and Quamme, 1986; Doubek and Armbrecht, 1989; Vincenzini et al., 1989; Wallis et al., 1993), others have used everted intestinal rings (Darmenton et al., 1989; Thompson et al., 1988; Ferraris and Vinnekota, 1995). As well, the method of expressing results is also important. Most studies have expressed uptake based on intestinal weight, and have therefore failed to take into account any potential age- and diet- associated changes in mucosal weight or surface area. The strain of the animals used, the ages of the animals, and the site of the intestine used may also differ between studies, and may explain the variability in the results.

The uptake of fructose has been studied in aging mice. Ferraris et al. (1993) showed that D-fructose uptake per milligram of tissue was higher in the jejunum of young as compared to old animals. The effect of diet on D-fructose uptake in aging animals was also examined (Ferraris et al., 1993). D-fructose uptake was also found to be higher in younger animals fed a high carbohydrate, low protein diet. No differences in D-fructose uptake were seen between young and old animals fed a no carbohydrate, high protein diet.

The uptake of fructose across the BBM is mediated by GLUT5, a sodium independent facilitative transporter (Vazquez et al., 1997). The transport of these sugars out of the enterocyte across the BLM occurs via the facilitative sodium-independent GLUT2 transporter (Thorens, 1996). In addition to its role as a BLM transporter, GLUT2 has recently been localized in the BBM, where it has been suggested to contribute to the uptake of sugars into the enterocyte (Kellett, 1997; Helliwell et al., 2000a; Helliwell et al., 2000b; Kellett and Helliwell, 2000).

Intestinal adaptation is a process by which the intestine changes both morphologically and/or functionally in response to alterations in environmental stimuli. Dietary manipulations have been shown to modify intestinal adaptation and nutrient absorption. Young animals fed a diet enriched in saturated fatty acids (SFA) have greater glucose uptake than do animals fed an isocaloric diet high in polyunsaturated lipids (PUFA) (Thomson et al., 1986; Thomson et al., 1987; Thomson et al., 1988). Similarly, adult rats fed SFA show increases in fructose uptake as compared to those fed PUFA (Perin et al., 1997).

The intestinal adaptive process may be compromised during the process of aging and may thereby contribute to malabsorption and malnutrition. Ferraris and Vinnekota (1995) showed in aging rats that adaptive increases in uptake in response to a high carbohydrate diet were reduced in old as compared with young animals, and the changes were limited to the more proximal regions of the intestine. In other studies, following a 3 day starvation period, animals were re-fed for 1 day and enzyme specific activities were measured. Upon re-feeding, the specific activities of lactase, sucrase and maltase demonstrated an exaggerated enzyme response in aged animals (Holt and Kotler, 1987; Holt et al., 1988). It is not known how the aging rat responds to diets high in PUFA or SFA, or if modifications in dietary lipids could be used to alter the expected age-associated changes in nutrient absorption.

The objectives of this study were to determine 1) the effects of aging on the *in vitro* uptake of fructose in rats; 2) the effect of feeding SFA versus PUFA; and 3) the molecular mechanisms of these age- and diet-associated changes.

7.2. Materials and Methods

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and the Council of the American Physiological Society, were observed in the conduct of this study. Thirty six male Fischer 344 rats, aged 1, 9 and 24 months were obtained from the National Institute of Aging colony and Harlan Laboratories, Maryland, D.C. Pairs of rats were housed at a temperature of 21°C, with 12 h of light and 12 h of darkness. Water and food were supplied *ad libitum*.

Animals were fed standard Purina® rat chow for one week, then fed one of two diets for a further two weeks: a semi-purified diet containing 20% (w/w) fat and enriched with either SFA or the same diet enriched with PUFA (Table 13 and 14). There were a total of 6 animals in each age and diet combination. The isocaloric semi-purified diets were nutritionally adequate, providing for all known essential nutrient requirements. Animal weights were recorded at weekly intervals.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labelled D-fructose was supplied by Amersham Biosciences Inc. (Baie d'Urfe, QC), and the unlabelled fructose by Sigma (St Louis, MO) respectively. The concentrations used were: 8, 16, 32, and 64 mM. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume.

Tissue preparation

The animals were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbitol, 240 mg/100 g body weight). The small intestine was removed from the ligament of Treitz to the ileocecal valve and rinsed with cold saline. This segment was then divided into thirds, with the proximal third considered to be the "proximal" small intestine, and the distal third considered to be the "distal" small intestine. The intestine was opened along its mesenteric border, and pieces of the proximal segment (jejunum) and the distal segment (ileum) were cut and mounted as flat sheets in the transport chambers. A 5 cm piece of each jejunal and ileal segment was gently scraped with a glass slide to determine the percentage of the intestinal wall comprised of mucosa. The chambers were placed in preincubation beakers containing oxygenated Krebs-bicarbonate buffer (pH 7.2) at 37 °C, and tissue discs were preincubated for 15 min to allow the tissue to equilibrate at this temperature. The rate of fructose uptake was determined from the timed transfer of the transport chambers to the incubation beakers containing [^{3}H]-inulin and ^{14}C-labelled fructose in oxygenated Krebs-bicarbonate (pH 7.2, 37°C). Preincubation and incubation chambers were mixed with circular magnetic bars at identical stirring rates, which were precisely adjusted using a strobe light. Stirring rates were reported as revolutions per minute (rpm). A stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer (Lukie et al., 1974; Westergaard and Dietschy, 1974; Westergaard and Dietschy, 1976).

Determination of uptake rates

After incubating the discs in labeled solutions for 6 min, the experiment was terminated by removing the chamber and rinsing the tissue in cold saline for approximately 5 s. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, placed on a glass slide, and dried overnight in an oven at 55°C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Lukie et al., 1974).

The rates of uptake of fructose were determined as nmol 100 mg tissue $^{-1}$ min $^{-1}$, nmol 100 mg mucosal tissue $^{-1}$ min $^{-1}$, and nmol cm $^{-2}$ serosal surface area min $^{-1}$, and nmol cm $^{-2}$ mucosal surface area min $^{-1}$. Because the relationship between uptake and fructose concentration was linear, the slopes of the lines were calculated and compared to determine statistical differences.

Morphology, messenger RNA and protein analysis

Tissue preparation

An additional 24 animals (4 in each of the 6 age/diet groups) were raised and sacrificed similarly as for the uptake studies. A 5 cm portion from each of the proximal jejunum and distal ileum was rinsed, quickly harvested, snap frozen in liquid nitrogen, and stored at –80°C for later mRNA isolation. Mucosal scrapings were harvested from the remaining proximal and distal small intestine, snap-frozen in liquid nitrogen, and stored at –80°C for subsequent isolation of BBM and BLM. For morphology and immunohistochemistry analysis, two 1cm pieces of proximal and distal small intestine were fixed in 10 % formalin.

Morphological Measurements

Morphometric data were obtained from hematoxylin and eosin stained paraffin sections. Vertical cross sections of the intestinal tissue were used to obtain measurements of villous height, villous width at one-half villous height, villous bottom width and crypt depth. The tissue block was then re-oriented 90° laterally and sections were taken to determine the villous depth at one-half villous height. Magnification was calibrated using a micrometer. Mucosal surface area was calculated as described previously (Thomson et al., 1987). The number of villi per millimeter of serosal length was measured in longitudinal and horizontal cross sections, then multiplied together to obtain the number of villi per square millimeter serosa. When this villous density was multiplied by villous surface area, the result was the mucosal surface area, expressed as square millimeters per square millimeter of serosa. At least 10 villi were assessed per section. The 2 following formulae were used (Thomson et al., 1987):

Villous surface area (μm^2/villus)= $(2 \times M \times H) + (2 \times M - A) \times D + (2 \times D) \times [(A-M)^2 + (H)^2]^{0.5} \times 1000$, where H=villous height, M=villous width at one-half height, A=villous bottom width, and D=villous thickness.

Mucosal surface area (mm^2/mm^2 serosa) = number of villi/mm^2 serosa x villous surface area (μm^2/villus)/1000

Messenger RNA abundance

The intestinal pieces were homogenized in a denaturing solution containing guanidinium thiocyanate, using The Fast Prep® cell disruptor (Savant Instruments Inc., Holbrook, New York.) Following addition of 2 M sodium acetate, a phenol chloroform extraction was performed. The upper aqueous phase containing the RNA was collected. RNA was precipitated with isopropanol overnight at -80°C, with a final wash with 70% ethanol. The concentration and purity of RNA was determined by spectrophotometry at 260 and 280 nm. Samples were stored at -80°C until use for Northern blotting.

Fifteen [15] ug of total RNA was fractionated by agarose gel electrophoresis and transferred to nylon membranes by capillary diffusion. RNA was fixed to the membranes by baking at 80°C for 2 hr. Northern blotting was performed used the DIG Easy Hyb ® method, according to the manufacturers protocol (Roche Diagnostics, Quebec, Canada).

The GLUT2 and GLUT5 plasmids were kindly donated by Dr. G.I. Bell, of the Howard Hughes Medical Institute, University of Chicago.

The density of the mRNA bands was determined by transmittance densitometry (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario). Quantification of the 28S ribosomal RNA units from the ethidium bromide stained membranes was used to account for loading discrepancies.

Protein analysis

Brush border membranes (BBM), basolateral membranes (BLM), and enteroctye cytosol were isolated from rat intestinal mucosal scrapings by differential centrifugation, and Ca^{2+} precipitation (Maenz and Cheeseman, 1986; Orsenigo et al., 1985; Orsenigo et al., 1987). Aliquots were stored at -80°C. For Western blotting, GLUT5 was measured in the BBM, and GLUT2 was measured in the BLM. Each group was represented in each blot that was probed. The results were expressed as a percentage of the total density on each blot, in order to normalize for differences in development. We had previously determined that 15ug of protein was sufficient for detecting a signal without saturating the detection system.

The protein concentration of the samples was determined using the Bio-Rad Protein Assay® (Life Science Group, Richmond, CA). Proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and were transferred to nitrocellulose membranes. Transfer efficiency was verified by Ponceau S (3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2, 7-naphtalenediasulfonic acid) staining of membranes, and by Coomassie Blue staining of gels. Membranes were blocked by incubation overnight in BLOTTO (Bovine Lacto Transfer Technique Optimizer) containing 5% w/v dry milk in Tween Tris Buffered Saline (TTBS) (0.5% Tween 20, 30mM Tris, 150 mM NaCl).

Membranes were washed in TTBS (3 x 10 min each) and subsequently probed with specific rabbit anti-rat antibodies to GLUT5 (Chemicon International, Temecula, California) and GLUT2 (Biogenesis, Poole, England) for two hours at room temperature. The antibodies were prepared in 5% dry milk in TTBS at a dilution of 1:500. Membranes were subsequently washed with TTBS to remove the residual unbound primary antibody, and were then incubated for 1 hr with goat anti-rabbit antibody (1:20000 in 2 % dry milk in TTBS) conjugated with horseradish peroxidase (HRP) (Pierce, Rockfort, Illinois, USA.

Membranes were washed again in TTBS to remove residual secondary antibody, and were briefly incubated with Supersignal® Chemiluminescent-HRP Substrate (Pierce, Rockfort, Illinois, USA). Membranes were exposed to X-OMAT AR films (Medtec, Burnaby, British Columbia) and the relative band densities were determined by transmittance densitometry using Bio-Rad Imaging Densitometer (Model GS-670, Imaging densitometer, Biorad Laboratory, Mississauga, Ontario).

Immunohistochemistry

Jejunal and ileal tissue was embedded in paraffin, and 4-5 micron sections were mounted on glass slides, dewaxed in xylene, and hydrated after incubation in a series of ethanol incubations. Slides were then incubated in hydrogen peroxide/methanol solution (20% - 50% H_2O_2, 80% methanol) for 6 min, rinsed in tap water and counterstained with Harris Hematoxylin (10 sec). Slides were then air-dried and the tissue was encircled with hydrophobic slide marker (PAP pen, BioGenex, California). After rehydration in phosphate buffered saline (PBS), the slides were incubated for 15 min in blocking reagent (20% normal goat serum) followed by primary antibody to GLUT5 for 30 min. Slides were then washed in PBS and incubated in LINK® and LABEL® according to manufacturers protocol. The solutions were subsequently incubated for 5 min in DAB®, rinsed in water, dehydrated in absolute ethanol, and cleared in xylene. Negative controls were processed on the same slide in an identical manner, excluding the incubation with the primary antibody. A Leitz Orthoplan Universal Largefield microscope and a Leitz Vario Orthomat 2 automatic microscope camera were used to photograph the slides. Chromagen staining was quantified using a Pharmacia LKB-Imagemaster DTS densitometer and Pharmacia Imagemaster 1D (Version 1.0) software (Amersham Pharmacia Biotechnology Inc, Piscataway, New Jersey, USA). Four villi per animal were quantified, and the results were normalized to the negative control values.

Expression of results

The results were expressed as mean ± standard error of the mean. Sigmastat (Version 1.0) was used to perform two–way analysis of variance (ANOVA) ($p<0.05$) in order to determine the effect of age and diet and any age*diet interactions. Individual differences between ages were determined using a Student-Neuman-Keuls multiple range test.

7.3. Results

Animal Characteristics

The rate of body weight change (grams per day) fell between 1, 9 and 24 months in rats fed SFA or PUFA (Figure 40). At each age the body weight decline was greater with SFA than with PUFA. Food intake was not influenced by the age of the rats, regardless of whether they were fed SFA or PUFA (data not shown).

Age and diet had no effect on jejunal or ileal tissue weight, mucosal weight or percentage of the intestinal wall comprised of mucosa (Table 15).

There were no differences in the mean values of the heights of the villi of the jejunum or ileum of rats aged 1, 9 or 24 months (data not shown). In animals fed SFA, the jejunal and ileal mucosal surface areas were lower at 9 and 24 months, as compared with 1 month (Figure 41). In those fed PUFA, both the jejunal and ileal mucosal surface area were lower at 24 as compared with 1 and 9 months. In the jejunum, diet had no effect on mucosal surface area at 1, 9, or 24 months (Figure 41). In the ileum, mucosal surface area was increased in 1 month old animals fed SFA as compared to PUFA. Diet did not affect ileal mucosal surface area at 9 or 24 months of age.

The age-associated decline in jejunal mucosal surface area occurred between 9 and 24 months in rats fed PUFA, and between 1 and 9 months in those fed SFA. Thus, feeding PUFA slowed the age-associated decline in the mucosal surface area of the jejunum and ileum. Thus, the surface area of the mucosa had to be taken into account when expressing the rate of uptake of fructose.

Fructose Absorption

When fructose uptake was expressed on the basis of the weight of the wall of the intestine (nmol 100 $mg^{-1}min^{-1}$), neither age nor diet significantly affected fructose uptake in the jejunum or ileum (Figure 42).

Similarly, when fructose uptake was expressed on the basis of the weight of the mucosa (nmol 100 mg $mucosa^{-1}min^{-1}$), neither age nor diet significantly affected fructose uptake in the jejunum or ileum. (Figure 43).

Similarly, when fructose uptake was expressed on the basis of the serosal surface area (nmol cm^{-2} min^{-1}), neither age nor diet significantly affected fructose uptake in the jejunum or ileum. (Figure 44).

When uptake was expressed on the basis of mucosal surface area, in animals fed SFA both the jejunal and ileal uptake of fructose was greater at 9 or at 24 months as compared with 1 month (Figure 45). In animals fed PUFA, both the jejunal and ileal uptake of fructose was higher at 24 months than at 9 or at 1 month. Diet influenced ileal fructose uptake. At 9 months, uptake was higher in animals fed SFA as compared to animals fed PUFA. In contrast, at 24 months uptake was higher in animals fed a PUFA diet as compared to animals fed SFA.

Transporter Protein Abundance and Immunohistochemistry

In animals fed SFA or PUFA, the jejunal and ileal abundance of GLUT5 determined by Western blotting was similar at 1, 9 and 24 months (Figure 46). Diet did not significantly affect jejunal or ileal GLUT5 protein abundance.

Jejunal and ileal villi were divided into 5 equal sections starting from the tip of the villi down to the crypt region. The abundance of GLUT5 protein was found to be evenly distributed along the crypt-villus axis (data not shown). The jejunal abundance of GLUT5 as determined by immunohistochemistry was not influenced by age or diet (Figure 47). In contrast, in SFA fed animals ileal GLUT5 was reduced at 9 and 24 months, when compared to 1 month. In PUFA fed animals the reduction in GLUT5 was evident only at 24 months when compared to 1 or 9 month old animals. At both 1 and 24 months, SFA fed animals had increased GLUT5 abundance when compared to PUFA fed animals. There was a significant interaction between age and diet (p=0.001).

The abundance of GLUT2 in the BLM was similar at 1, 9 and 24 months in animals fed SFA or PUFA (data not shown).

Transporter mRNA Expression

The expression of GLUT5 as well as GLUT2 in the jejunum was similar at 1, 9 and 24 months in animals fed SFA or PUFA (data not shown).

7.4. Discussion

Variations in dietary lipids influence the changes in intestinal surface area seen with aging. Animals fed SFA exhibited a reduction in surface area at both 9 and 24 months (Figure 41). This decrease in jejunal surface area seen with aging is delayed by feeding PUFA, as animals fed this diet showed reductions in surface area only at 24 months of age. In addition, feeding PUFA prevents the age-associated decline in ileal mucosal surface area seen with SFA. This signifies that the intestine of the old rats remains capable of adapting its morphology in response to dietary lipid manipulations. This observation also stresses the point that failure to take into account the animal's diet may lead to errors in the interpretation of the effects of aging on the morphology of the intestine. Finally, the rate of decline in jejunal and ileal mucosal surface area seen in rats fed SFA can be slowed by feeding PUFA.

The simplest way of expressing the rate of *in vitro* uptake of nutrients is on the basis of the weight of the full thickness of the wall of the intestine. However, if an experimental manipulation alters the weight of the intestine or the mucosal surface area, then there may be variations in the rate of nutrient uptake which are understandable in the light of there simply being more mucosal tissue or a greater surface area. For this reason, where there are treatment-associated variations in mucosal mass or the surface area of the villous membrane, as was the case in this study (Table 15 and Figure 41), then it is more appropriate to express uptake on the basis of mass of the transporting mucosal tissue or the mucosal surface area. Clearly, no significant differences in fructose uptake are seen when expressed on the basis of intestinal weight (Figure 42), mucosal weight (Figure 43) or serosal weight (Figure 44). However, significant increases in jejunal and ileal fructose uptake with both the SFA and PUFA diet are seen when expressed on the basis of mucosal surface area (Figure 45). Thus the ability to show an effect of age or diet on fructose uptake depends on the way in which the rate of uptake is expressed.

Previous studies have shown that fructose uptake is increased in young rats fed SFA as compared to PUFA (Perin et al., 1997). Our results confirm these findings in 9 month old animals. However, in the older 24 month animals, the diet effect appears to be reversed, as fructose uptake is increased with PUFA as compared to SFA. This suggests that dietary lipids affect the absorption of nutrients differently depending on the age of the animal. This finding is in agreement with other work (Ferraris et al., 1993; Holt and Kotler, 1987; Holt et al., 1988) that shows a reduction in adaptive capabilities in response to diet with aging. Indeed, the results of this study demonstrate that the ability of SFA to increase fructose uptake is impaired with aging. Ferraris and Vinnekota (1995) noted that the effect of dietary changes in aged mice was limited to the proximal small intestine, but the effect of dietary lipids on glucose uptake in aged rats occurs in both the jejunum and ileum (Figures 42-45). Thus, aging modifies the adaptability of the intestine in response to dietary manipulation. Clearly, the influence of a manipulation that results in intestinal adaptation in young rats does not necessarily apply in older animals.

Despite the decreases in the surface area of the intestine seen with aging, fructose uptake, when expressed on the basis of mucosal surface area, is increased with age in animals fed PUFA or SFA (Figure 45). The aged intestine is therefore able to maintain a high absorptive capability, despite a reduction in absorptive surface area. However, the changes in uptake seen with aging or with diet are not solely explained by alterations in mRNA and protein abundance of the fructose transporters: PUFA or SFA, however, show increases in fructose uptake without concomitant changes in GLUT5 abundance. In fact, in the ileum, reductions in GLUT5 abundance as determined by immunohistochemistry are in contrast to the increases in uptake seen with aging.

In models of diabetes, a "recruitment" of transporters in the lower part of the villi results in active transport occurring in this area, and a resultant increase in glucose transport (Burant et al., 1994). As most intestinal glucose uptake occurs in the upper third of the villi (Thomson et al., 1994), a redistribution of GLUT5 to this area could explain altered uptake. However, in fact GLUT5 was evenly distributed along the crypt-villous axis (data not shown), and therefore a change in the distribution of GLUT5 does not explain altered uptake.

One could speculate that changes in the fluidity of the BBM as a result of aging could also explain the apparent uncoupling of transport to both RNA abundance and protein abundance. There are reductions in the membrane fluidity of the BBM isolated from 117 week old Fischer 344 rats as compared to younger animals (Brasitus et al., 1984). Similarly, the fluorescence polarization technique used by Wahnon and coworkers (1989) showed reductions in membrane fluidity in 19 month old rats when compared to 1 and 9 month old rats. Indeed, a study done using chickens demonstrated that reductions in membrane fluidity, as a result of changes in BBM lipid content, may be involved in the decrease in D-glucose uptake observed during post-hatching development (Vazquez et al., 1997). With aging, declines in membrane fluidity are associated with increases in uptake (Meddings et al., 1990). Alterations in the fluidity of the BBM as a result of dietary lipid manipulations might explain the uncoupling of transport to RNA abundance and protein abundance. Altering the fatty acid composition of the diet results in changes in the phospholipid content of the BBM of enterocytes (Keelan et al., 1997). It is reasonable to speculate that changing the dietary lipids may have altered BBM fluidity and therefore GLUT5 function in the older animals. We may further speculate that transporters such as GLUT5 or GLUT2 may reside in specialized lipid rafts, and local changes in membrane fatty acids may be responsible for alterations in function. Further research is needed to fully characterize the effects of changes in raft-associated membrane lipids on intestinal nutrient transport.

It has been proposed that GLUT2 is present in the BBM, as well as the BLM, and transports glucose and fructose into the cell via facilitated diffusion

(Kellett, 1997; Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Au et al., 2002; Affleck et al., 2003; Helliwell et al., 2003; Gouyon et al., 2003). The changes in uptake seen in this study may, therefore, be paralleled by increases in BBM GLUT2 protein. However, GLUT2 appears to only play a significant role in sugar uptake in the presence of high luminal sugar concentrations, such as those found after a high sugar meal or following a sugar bolus (Kellett, 1997; Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Helliwell et al., 2003; Gouyon et al., 2003). In this study we did not detect any GLUT2 protein in the BBM (data not shown). This is not surprising, as these animals were not fed diets high in sugar, nor were they given a sugar bolus. Therefore, we do not believe that GLUT2 in the BBM played a significant role in intestinal sugar uptake in this model and is not likely to explain the changes in uptake that were observed.

In conclusion, despite age-related reductions in mucosal surface area, intestinal fructose uptake per unit mucosal surface area increases with age. Changes in fructose uptake were not paralleled by changes in BBM GLUT5 or BLM GLUT2 protein abundance. The intestinal adaptive response to PUFA or SFA enriched diets is also influenced by aging, suggesting that the results obtained from studies of intestinal adaptation in young populations cannot necessarily be generalized to older populations. Further studies are required to determine the potential signals involved in the age-related changes in intestinal fructose uptake.

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Table 13. Fatty acid composition of the semi-synthetic diets

FATTY ACID (% of total)	Diet enriched with saturated fatty acids (SFA)	Diet enriched with polyunsaturated fatty acids (PUFA)
14:0	1.8	0.1
14:1n9	0.0	0.0
15:0	0.2	0.0
16:0	21.8	5.5
16:1n7	0.0	0.1
18:0	61.1	5.2
18:1n9	2.8	16.3
18:1n7	0.1	0.2
18:2n6	9.6	69.2
18:3n3	0.0	0.8
18:3n6	0.0	0.1
18:4n3	0.0	0.0
19:0	0.2	0.0
20:0	1.5	0.2
20:1n9	0.0	0.3
20:1n7	0.1	0.3
20:2n6	0.1	0.2
20:3n9	0.1	0.2
20:3n6	0.0	0.0
20:4n6	0.0	0.0
20:5n3	0.0	0.3
22:0	0.0	0.4
22:1n9	0.0	0.0
22:1n7	0.3	0.3
22:4n6	0.0	0.1
22:5n6	0.0	0.0
22:5n3	0.1	0.1
24:0	0.1	0.1
22:6n3	0.1	0.0

Safflo® sunflower oil was obtained from Unico, Concord, Ontario, Canada
Beef Tallow was obtained from Canamera Foods, Edmonton, Alberta, Canada

Table 14. Macronutrient composition of the semi-synthetic diets

INGREDIENTS	CONCENTRATION (g/kg diet)
Fat	200.00
Cornstarch	378.00
Casein	270.50
Non-nutritive fiber	80.00
Vitamin mix*	10.00
Mineral mix#	50.00
L-methionine	2.50
Choline	2.75
Inositol	6.25

- * A.O.A.C. vitamin mix (Teklad Test Diets, Madison, WI) provided the following per kilogram of complete diet: 20 000 IU of vitamin A; 2000 IU of vitamin D; 100 mg of vitamin E; 5 mg of menadione; 5 mg of thiamine-HCl; 8 mg of riboflavin; 40 mg of pyridoxine-HCl; 40 mg of niacin; 40 mg of pantothenic acid; 2000 mg of choline; 100 mg of myoinositol; 100 mg of p-aminobenzoic acid; 0.4 mg of biotin; 2 mg of folic acid; and 30 mg of vitamin B12.
- # Bernhart Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH) was modified to provide 77.5 mg of Mn and 0.06 mg Se per kilogram of complete diet.
- Safflo® sunflower oil was obtained from Unico, Concord, Ontario, Canada Beef Tallow was obtained from Canamera Foods, Edmonton, Alberta, Canada

Table 15. Effect of Age and Diet on Intestinal Weight

	1 month		9 months		24 months	
	SFA	PUFA	SFA	PUFA	SFA	PUFA
Tissue weight (mg/cm)						
Jejunum	8.4±0.9	10.6±1.4	12.5±0.5	11.2±0.8	13.6±2.1	10.5±1.1
Ileum	6.8±0.6	9.4±0.8	10.4±1.9	7.6±1.1	8.5±0.9	5.4±1.1
Mucosal weight (mg/cm)						
Jejunum	4.2±0.7	5.5±0.9	6.3±0.6	5.9±1.0	7.2±1.5	5.6±0.8
Ileum	3.1±0.5	4.7±0.7	5.6±1.1	3.9±1.0	4.2±0.7	2.9±0.7
% Mucosa						
Jejunum	48.1±3.7	49.7±6.2	50.6±3.7	51.8±6.2	51.4±3.6	51.2±3.4
Ileum	45.6±5.0	49.9±5.3	51.4±3.6	48.8±5.4	49.3±4.0	42.8±8.9

Mean ± SEM
No statistically significant differences were detected by 2 way ANOVA

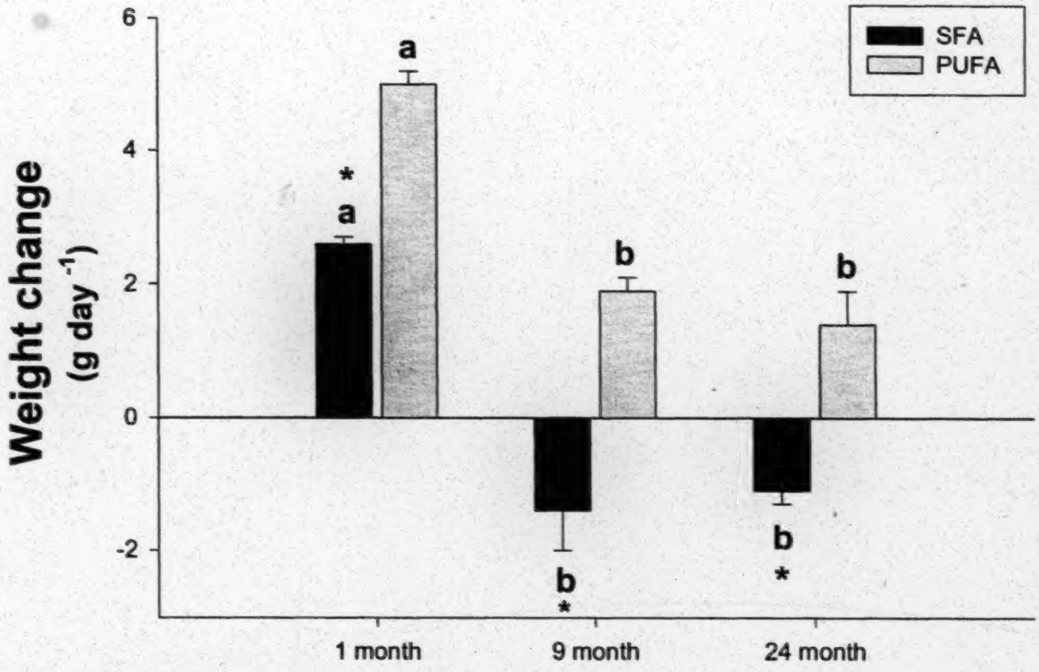


Figure 40. The effect of age and dietary lipids on body weight change.
Values are mean±SEM.
Different letters indicate a significant age effect
* indicates a significant diet effect
Statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)

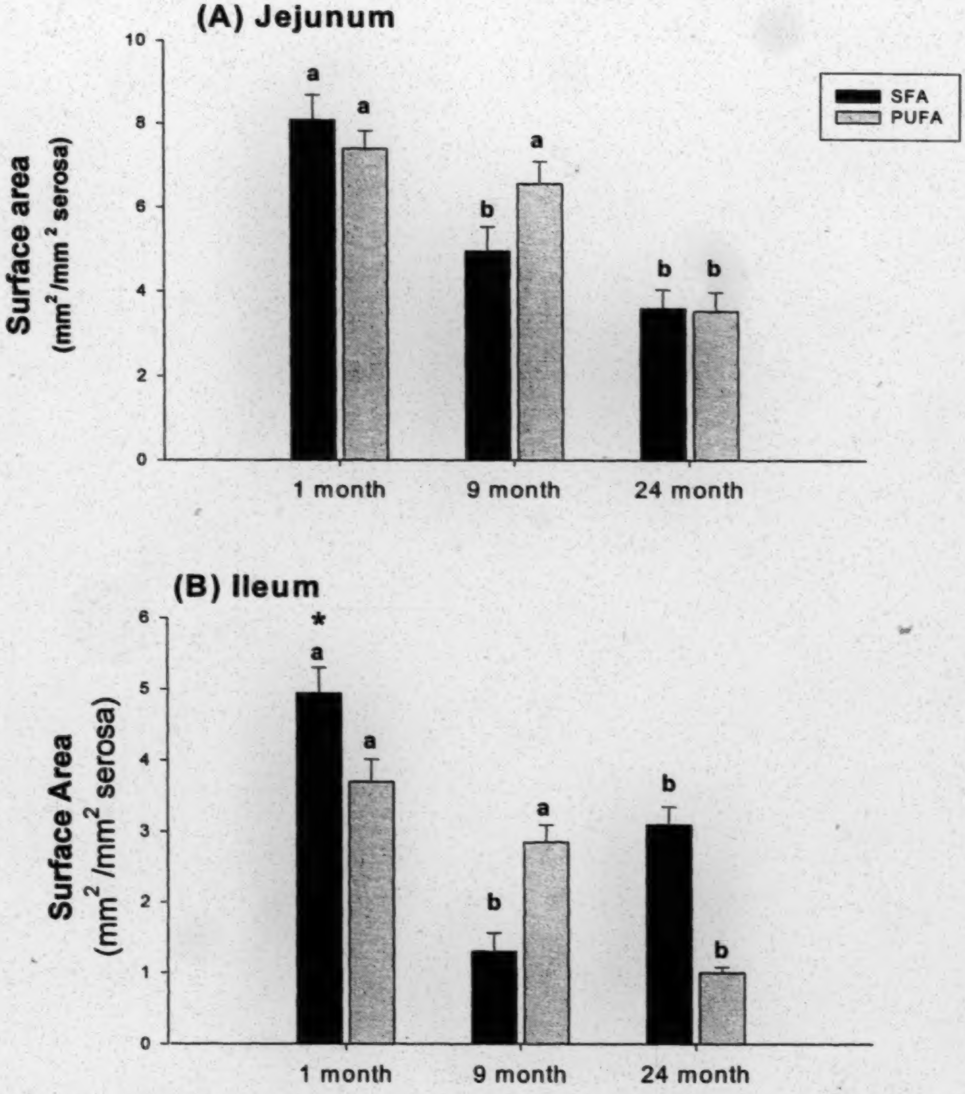
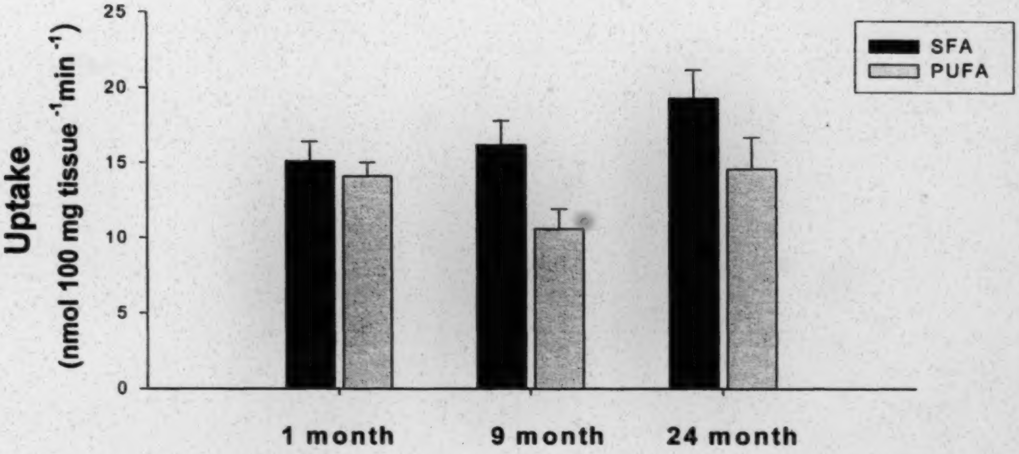


Figure 41. The effect of age and dietary lipids on the mucosal surface area of the small intestine. Values are mean±SEM.
Different letters indicate a significant age effect
* indicates a significant age*diet interaction
Statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)

(A) Jejunum



(B) Ileum

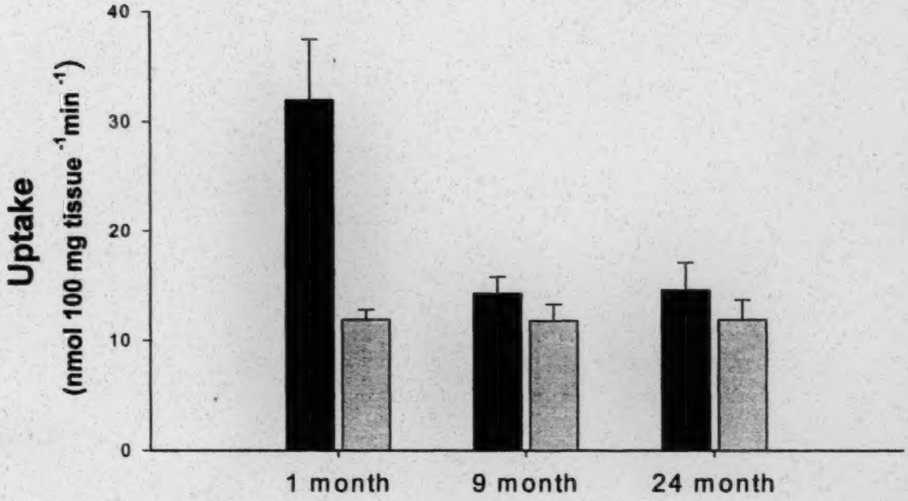
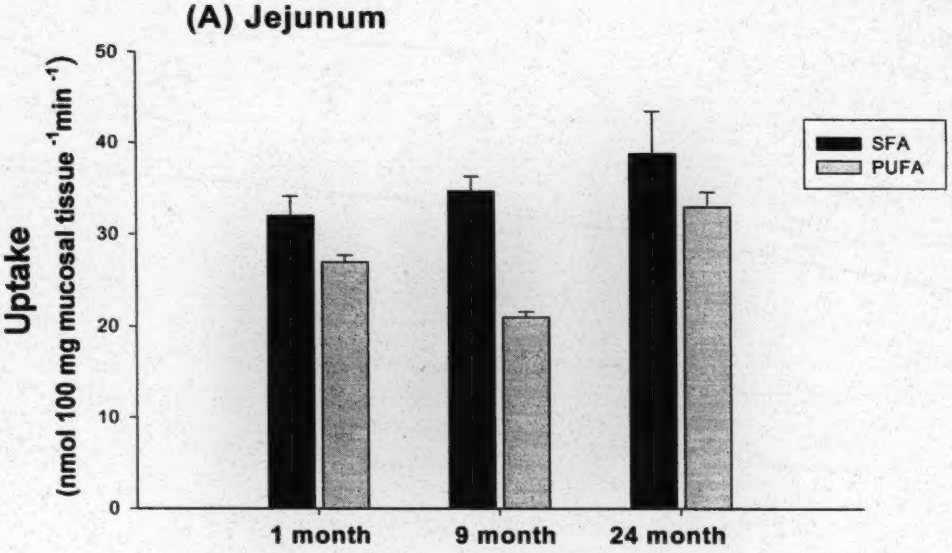


Figure 42. The effect of age and dietary lipids on D-fructose uptake expressed on the basis of intestinal weight. Values are mean±SEM.
No statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)



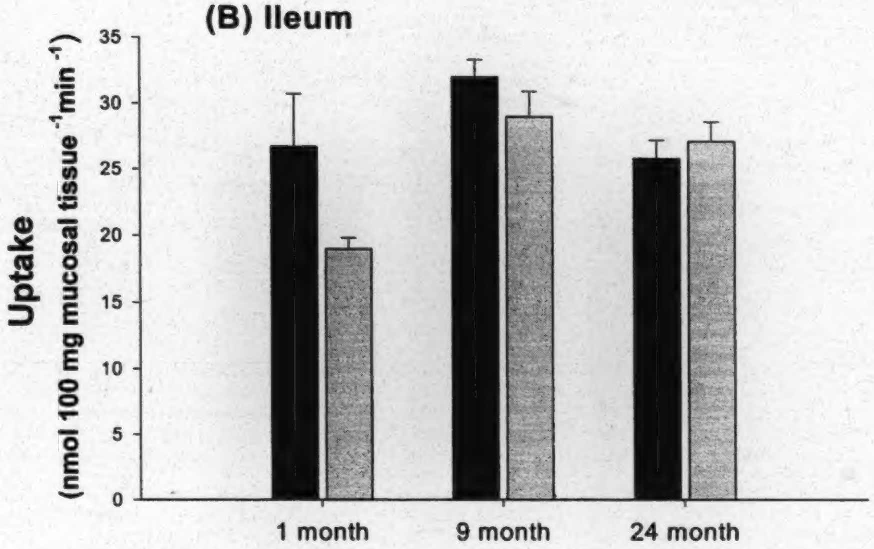


Figure 43. The effect of age and dietary lipids on the uptake of D-fructose expressed on the basis of mucosal weight. Values are mean±SEM.
No statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)

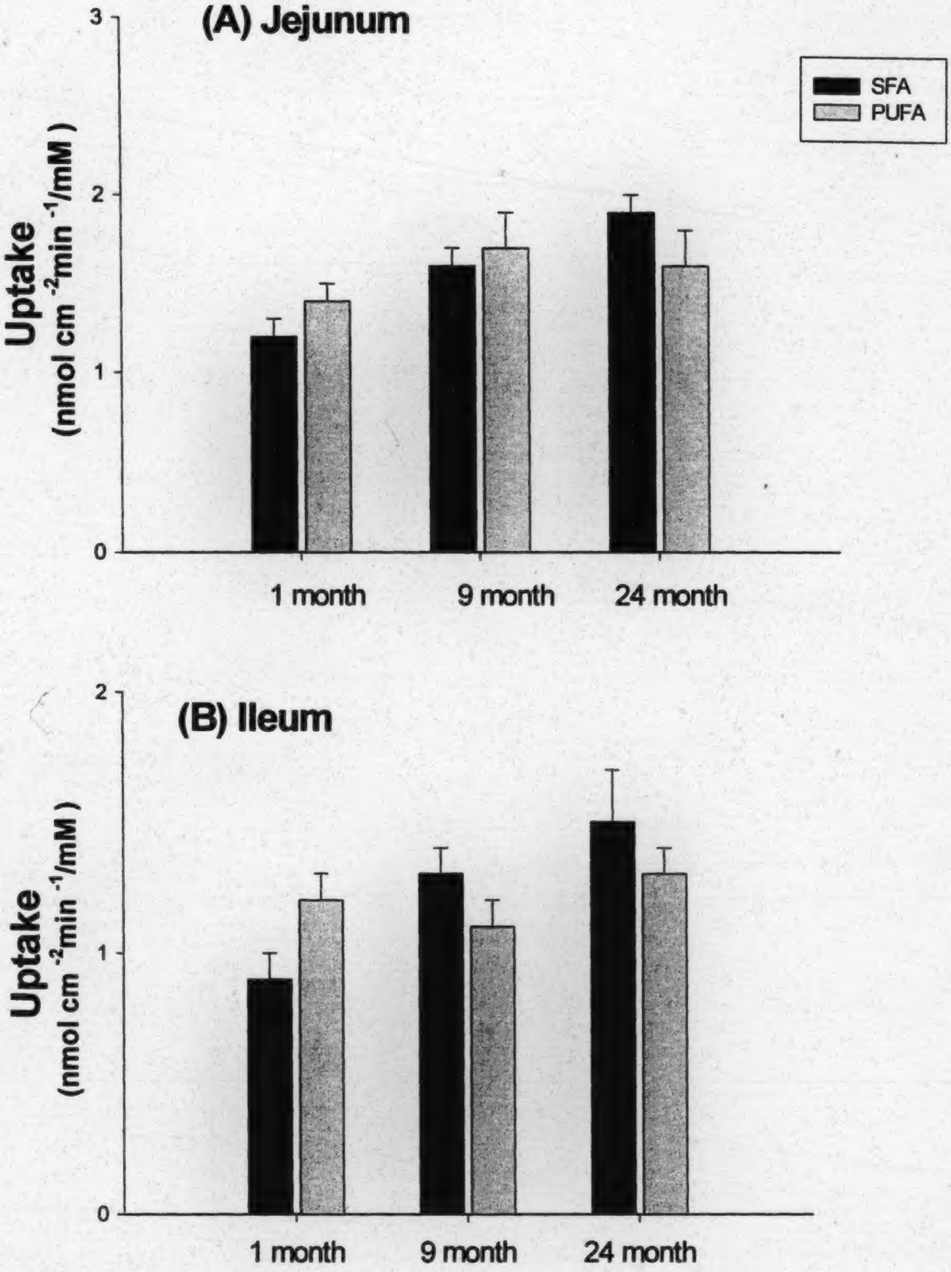


Figure 44. The effect of age and dietary lipids on the uptake of D-fructose expressed on the basis of serosal surface area. Values are mean±SEM.
No statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)

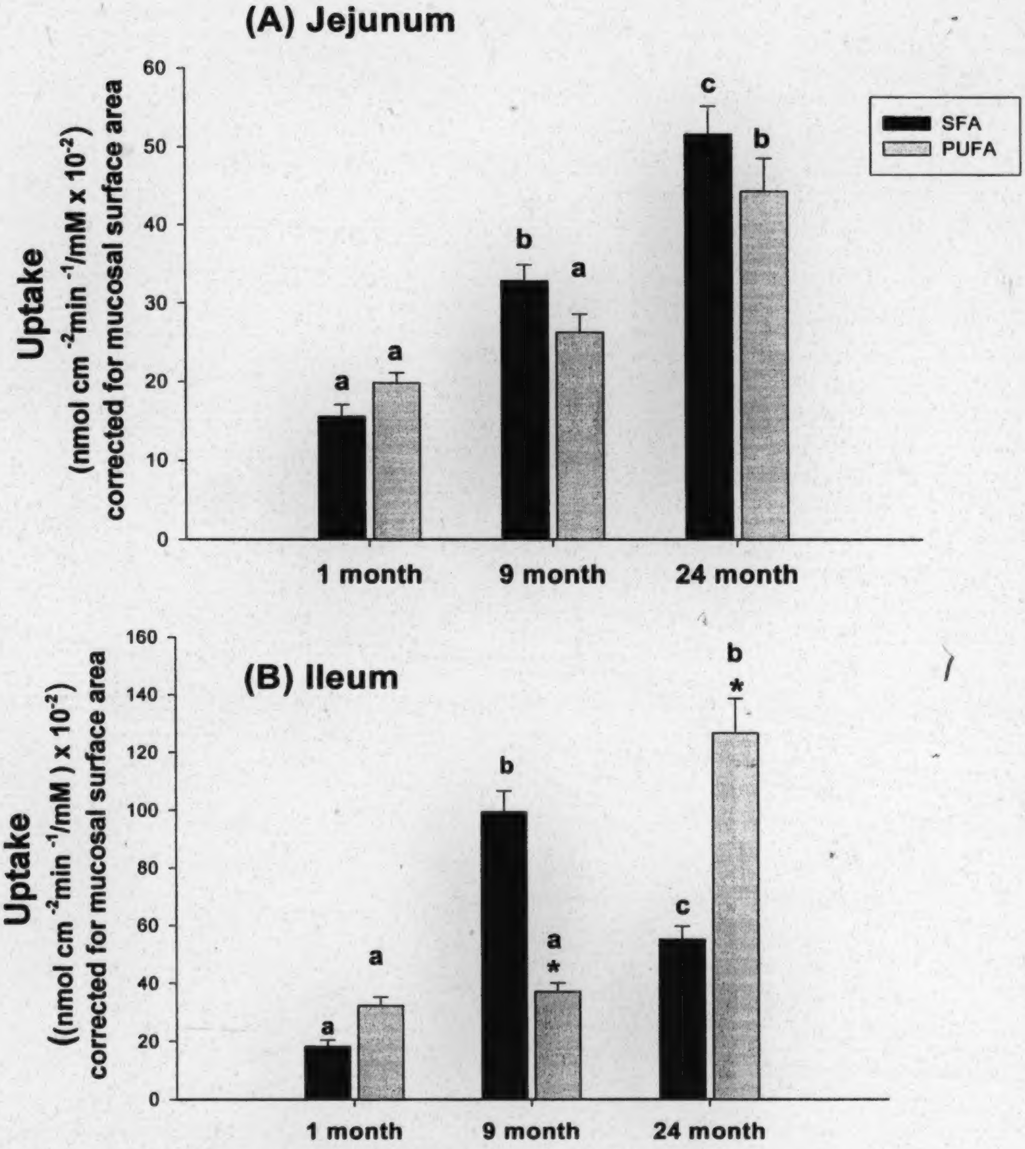


Figure 45. The effect of age and dietary lipids on the uptake of D-fructose expressed on the basis of mucosal surface area. Values are mean±SEM.

Different letters indicate a significant age effect

* indicates a significant age*diet interaction

Statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)

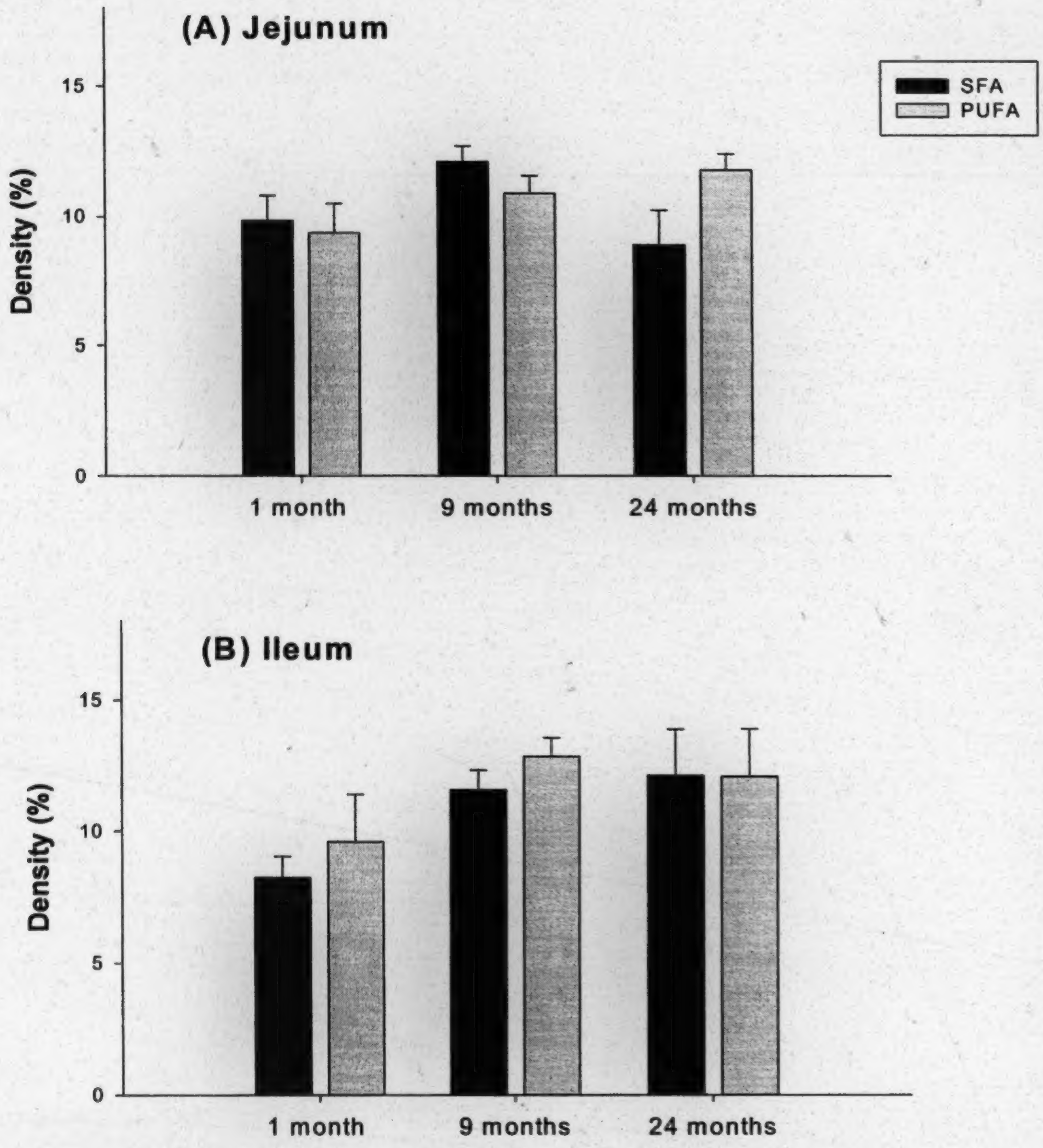


Figure 46.The effect of age and dietary lipids on GLUT5 protein abundance. Values are mean±SEM.

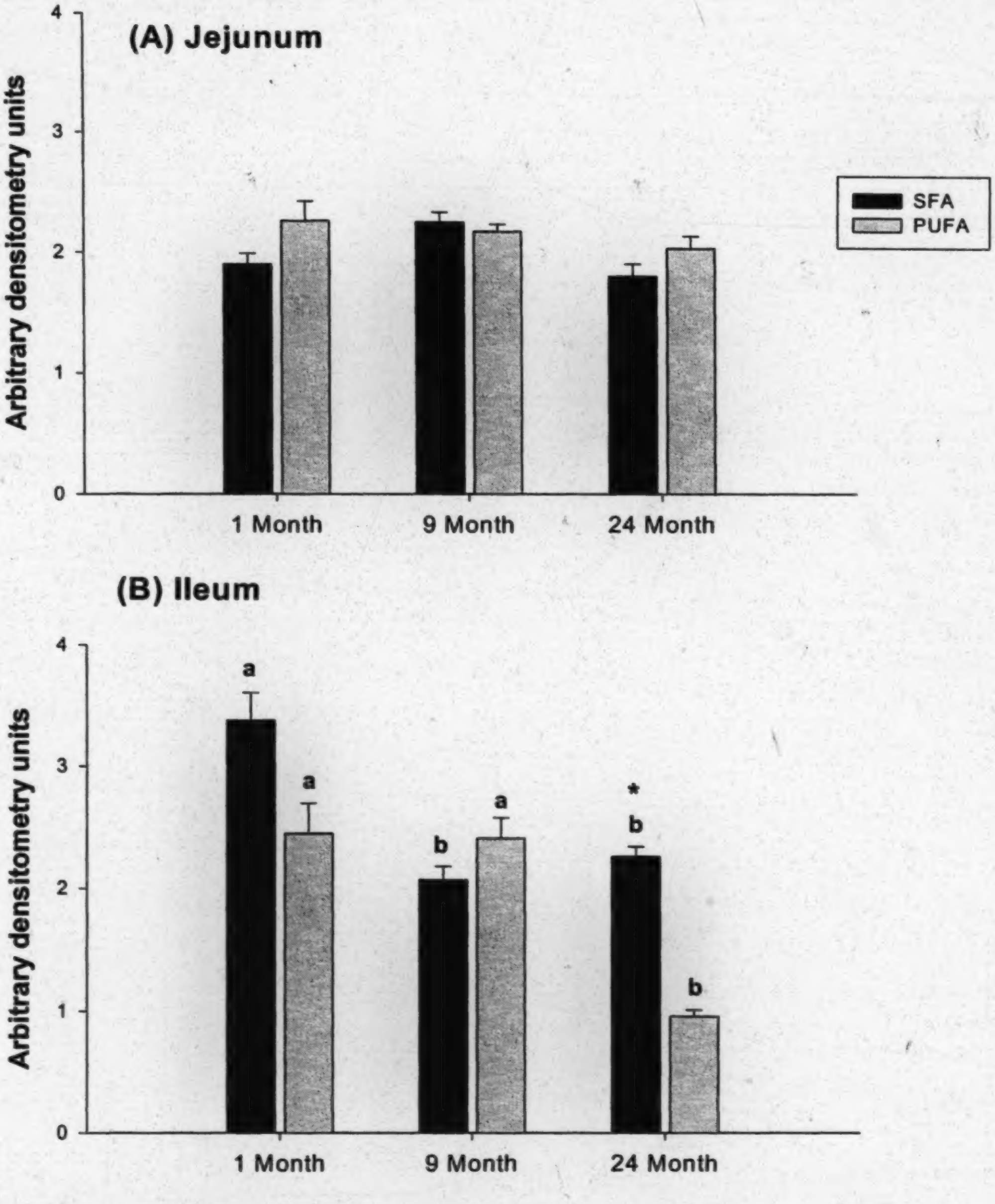


Figure 47. The effect of age and dietary lipids on GLUT5 abundance as determined by immunohistochemistry. Values are mean±SEM.
Different letters indicate a significant age effect
* indicates a significant age*diet interaction
Statistically significant differences were determined using a 2 way ANOVA ($p<0.05$)

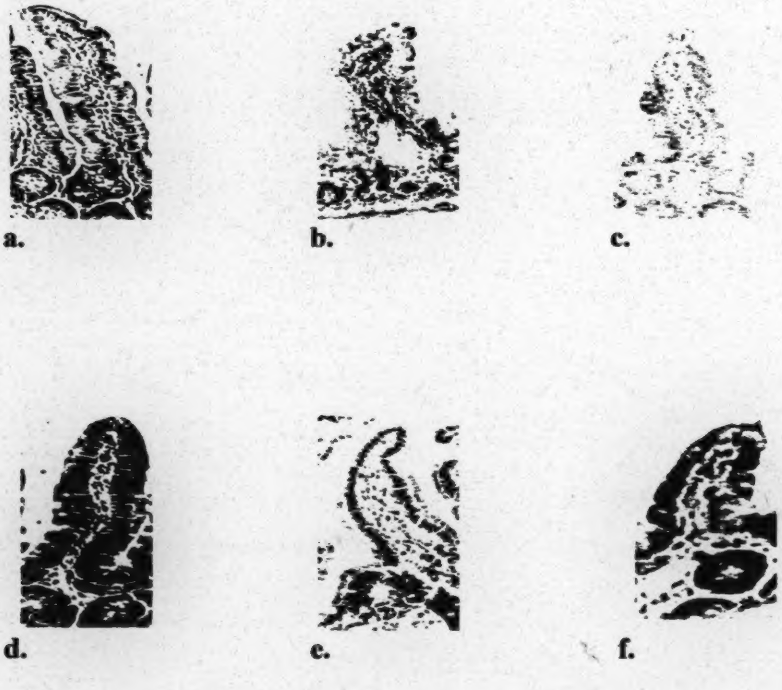


Figure 48. GLUT5 immunohistochemistry on ileal sections from a) 1 month old rats fed PUFA; b) 9 month old rats fed PUFA; c) 24 month old rats fed PUFA; d) 1 month old rats fed SFA; e) 9 month old rats fed SFA; and f) 24 month old rats fed SFA

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8. A COMBINATION OF DEXAMETHASONE AND GLP-2 INCREASE INTESTINAL MORPHOLOGY IN SUCKLING RATS

8.1. Introduction

Premature infants may have compromised intestinal function. Stimulating intestinal nutrient transport by administering a trophic agent may improve the nutritional status of the infant. Glucocorticosteroids have been shown to accelerate the digestive and transport function of neonatal rats (Moog et al., 1971; Galand, 1989; Solomon et al., 2001), but also increase the intestinal absorption of sugars and lipids in adults (Thiesen et al., 2002; Thiesen et al., 2003). GLP-2 is an intestinotropic hormone that induces intestinal adaptation in parenterally fed rats with short bowel syndrome (Martin et al., 2004). Vascular infusions of GLP-2 increase *in vivo* sugar absorption in adult rats (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998), and the combination of GLP-2 and dexamethasone given subcutaneously to sucklings increases lipid uptake later in life (Iordache et al., 2005). Although GLP-2 increases intestinal morphology and BBM enzymes in neonatal pigs (Petersen et al., 2001; Petersen et al., 2002), the effect of GLP-2 on intestinal sugar absorption in young animals has not been studied. It is also possible that GLP-2 and DEX may have an additive effect on nutrition absorption in suckling animals, as glucocorticoids have a permissive effect on hormones, such as GLP-2, which act on G-protein coupled receptors and increase adenylate cyclase (Michel et al., 1994; Meier, 1997).

Some experimental manipulations in early life will have a late effect, altering the ontogeny of the intestine. For example, variations in the lipid composition of the maternal diet influences nutrient uptake in the offspring and these effects persist into the post-weaning period (Perin et al., 1997; Jarocka-Cyrta et al., 1998; Perin et al., 1999). For example, feeding rat dams a diet with a high n6/n3 ratio results in offspring with higher rates of glucose uptake when compared to offspring whose dams were fed a diet with a low n6/n3 ratio. While early dietary exposure clearly influences intestinal transport, it is not known if hormones like glucocorticosteroids and GLP-2 given during the suckling period have a later effect in weanlings.

Accordingly, this study was designed to determine 1) the effect of GLP-2, DEX, and GLP-2+DEX on *in vitro* intestinal sugar uptake in suckling rats; 2) if these changes in sugar uptake are due to variations in the intestinal morphology or mass; and 3) if GLP-2, DEX, or GLP-2 + DEX have a late effect on sugar uptake in the weanlings.

8.2. Materials and Methods

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and by the Council of the American Physiological Society, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Alberta. Eight nursing Sprague Dawley rats with 64 suckling offspring were obtained from Bio Science Animal Services, University of Alberta. The suckling rats were randomized into four groups which received treatment with GLP-2, DEX, GLP-2 plus DEX, or Placebo. All animals received treatment for 10 days starting with the 11th day from time of their delivery. GLP-2 was administrated in a dose of 0.1 μg/g body weight/day subcutaneously [sc] twice daily at 7am and 7 pm. DEX was administrated in a dose of 0.128 μg/g body weight/day sc once daily at 7 pm. The regimen used for GLP-2 + DEX group was GLP-2 0.1 μg/g body weight/day sc twice daily at 7am and 7 pm, plus DEX 0.128 μg/g body weight/day sc once daily at 7 pm. The placebo group received 0.9% saline in a volume equal to the volume of GLP-2 administrated daily per rat sc, twice daily at 7 am and 7 pm. Dexamethasone was purchased from Sigma-Aldrich Canada Ltd (Oakville, ON), and rat GLP-2 was obtained from American Peptide Company (Sunnyville, CA).

There were eight animals in each group. Following treatment, all sucklings were sacrificed for uptake studies at 21 days of age, and all post-weaning animals ("weanlings") were sacrificed for the uptake studies at 7 weeks of age.

The animals were housed at a temperature of 21°C, and were exposed daily to 12 hours of light and 12 hours of darkness. During the suckling period, the offspring received only the dam's milk. The weanlings were housed in pairs. Their water and food were supplied *ad libitum*. The dams and the weanlings were fed standard rat chow, PMI #

5001 (Nutrition International LLC, Brentwood, MO, USA). The diets were nutritionally adequate, providing for all known essential nutrient requirements. Body weights were recorded at the time of weanling and then weekly for the next four weeks.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labeled probes included glucose (2-64 mM) and fructose (4-64 mM). The labeled and unlabeled probes were supplied by Amersham Biosciences Inc. (Baie d'Urfe, PQ) and Sigma (St. Louis, MO), respectively. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume (Westergaard and Dietschy, 1974).

Tissue preparation

Eight animals per treatment group were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbital, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with 150 ml cold saline. The intestine was divided into two parts: the proximal half of the intestine beginning at the ligament of Treitz was termed the "jejunum", and the distal half was termed the "ileum". A 2 cm piece of each segment of jejunum and ileum was gently scraped with a glass slide. The mucosal scrapings and the remaining wall of the intestine were dried overnight in an oven at 55°C. The percentage of the intestinal wall comprised of mucosa was calculated. The remaining intestine was everted, and cut into small rings of approximately 2-4 mm each. These intestinal rings were immersed in pre-incubation beakers containing Krebs's buffer (pH 7.2) at 37°C, bubbled with oxygen plus bicarbonate (O_2-CO_2, 95:5 by volume), and were allowed to equilibrate for 5 minutes (Perin et al., 1997). Uptake was initiated by the timed transfer of the tissue rings from the pre-incubation buffer to a 5 ml plastic vial containing [^{3}H]-inulin and ^{14}C-labelled sugars in Kreb's buffer bubbled with oxygen plus bicarbonate that had been equilibrated to 37°C in a shaking water bath. The intestinal rings were incubated in the sugar substrates for 5 minutes.

Determination of uptake rates

The rate of uptake of sugar was terminated by pouring the vial contents onto filters on an Amicon vacuum filtration manifold that was maintained under suction, followed by washing the intestinal rings three times with ice-cold saline. The tissue rings were placed on a glass slide, and were dried overnight in an oven at a constant temperature of 55°C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Westergaard and Dietschy, 1974). The rates of sugar uptake were determined as nmol of substrate absorbed per 100 mg dry weight of the whole intestinal wall per minute (Jd, nmol •100 mg tissue $^{-1}$• min $^{-1}$). Because the relationship between glucose uptake and concentration was curvilinear, the kinetic constants maximal transport rate (Vmax) and apparent Michaelis affinity constant (Km) were calculated by non-linear regression using the SigmaPlot program (Jandel Scientific, San Rafael, California, USA). This method is often used to estimate kinetic constants (Meddings and Westergaard, 1989; Fingerote et al., 1994). We also performed three linear transformations of the data (Lineweaver-Burke, Wolfee, Eadie-Hofstee) to confirm the results, as each method of estimation may over- or under-estimate the value of Vmax or Km (Thomson and Dietschy, 1977; Thomson, 1979a; Thomson 1979b; Thomson, 1981). Because fructose uptake was linear over the range of concentrations used (4-64 mM), the slopes of the lines were calculated and compared to determine statistically significant differences.

Morphological analysis

In order to determine the morphological characteristics of the intestine, a vertical section was prepared from the jejunum and from the ileum. Hematoxylin and eosin-stained slides were prepared from paraffin blocks. Crypt depth, villous height, villous width, villous width at half height, and cell density were measured using the program MetaMorph 5.05r (Universal Imaging Corporation, Downingtown, PA, USA). The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of four animals in each group. All morphological measurements were made by the same trained technician who was blinded to the different treatment groups.

Statistical analyses

The results obtained were expressed as mean ± standard error of the mean. The statistical significance of the differences between the four groups was determined by one way Analysis of Variance (ANOVA), with Student-Neuman-Keuls post-hoc analysis. Statistical significance was accepted as $p \leq 0.05$.

8.3. RESULTS

Body and Intestinal Weights, and Villous Morphology

Sucklings:

Injecting GLP-2, DEX or GLP-2 + DEX into the suckling animals for 10 days had no effect on the suckling animal's body weight (Figure 49). In contrast, the mean body weight of the weanlings injected six weeks previously with DEX was approximately 25% lower than in the control animals. When GLP-2 was given with DEX, the decrease in body weight in the weanlings given DEX was not observed.

Giving GLP-2, DEX, or GLP-2 + DEX to the suckling rats had no influence on the jejunal or ileal weights, weight of the mucosa, or percentage of the intestinal wall comprised of mucosa (data not shown). In contrast, in weanlings the total ileal weight was increased from 7.6 ± 0.8 mg/cm in controls to 10.1 ± 0.8 mg/cm ($p < 0.05$) in those given GLP-2 (Figure 50). Neither DEX nor GLP-2 + DEX influenced the intestinal weights of weanlings.

GLP-2 increased the crypt depth in the jejunum of sucklings, whereas DEX as well as GLP-2 + DEX increased the jejunal villous height, width at the villous base, and crypt depth (Table 16). In the ileum of sucklings, GLP-2 and DEX alone had no effect, whereas GLP-2 + DEX increased all the morphological parameters of the intestine (Table 16).

Weanlings:

In the jejunum of weanlings, GLP-2, DEX, and GLP-2 + DEX increased the distance between cells, indicating an increase in enterocyte size, and GLP-2 + DEX also increased the villous height (Table 17). In the ileum of weanlings, GLP-2 alone had no effect, DEX increased the villous height and cell size, and GLP-2 + DEX decreased the villous width, crypt depth, distance between villi, and distance between cells (Table 17).

Sugar uptake

Sucklings:

In suckling animals, fructose uptake was not affected by treatment with GLP-2, DEX or GLP-2+DEX when compared to controls (Table 18). The Vmax for jejunal glucose uptake was increased by DEX+GLP-2 (Wolfee and Eadie-Hofstee plot) and by GLP-2 (Wolfee plot) and DEX (Wolfee plot) (Table 19). In the ileum, the Vmax was reduced by DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot). The Km in the jejunum was increased by GLP-2+DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot) (Table 19). The effect of GLP-2 on the Km was inconclusive as the Lineweaver Burke and the Eadie-Hofstee plot showed decreases with GLP-2, while the Wolfee plot showed an increase. Similarly, DEX decreased the Km (Lineweaver Burke plot) while the Km was increased when the Wolfee plot was used.

Weanlings:

In weanling animals, fructose uptake was not affected by treatment with GLP-2, DEX or GLP-2+DEX (Table 18). The Vmax for jejunal glucose uptake was increased by GLP-2+DEX (Wolfee and Eadie-Hofstee plot) (Table 20). However, when the Lineweaver Burke plot was used the Vmax was reduced by GLP-2+DEX. In the ileum, the Vmax was increased by DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot) and by GLP-2+DEX (Eadie-Hofstee plot). The Km for jejunal glucose uptake was increased by DEX and GLP-2+DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot) and by GLP-2 (Wolfee plot). In the ileum, the Km was increased by DEX (Sigmaplot, Lineweaver Burke, Wolfee and Eadie-Hofstee plot) (Table 20).

8.4. DISCUSSION

Based on the literature on adult animals (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998), we had anticipated that the Vmax for glucose uptake into the jejunum and ileum of suckling animals would be increased with

GLP-2 and with DEX. We have determined the Vmax and Km for glucose uptake from a curvilinear plot, and have also performed three linear transformations of the data (Lineweaver Burke, Eadie-Hofstee, Wolfee), as alternate methods of determining these kinetic parameters. Using a curvilinear plot as well as a linear transformation (Lineweaver Burke), our data shows that none of the treatments significantly affected the value of the Vmax for glucose uptake (Table 19). However, there were increases in the Vmax in the jejunum with GLP-2 and DEX when the Wolfee plot was used, and increases with GLP-2+DEX when the Wolfee and the Eadie-Hofstee plot were used. In contrast, DEX significantly decreased the Vmax in the ileum when the data was assessed by linear transformation (Lineweaver-Burke, Wolfee, Eadie-Hofstee). Fructose uptake was also largely unaffected by treatment with DEX, GLP-2 or GLP-2+DEX (Table 18). The lack of changes in intestinal sugar uptake seen in sucklings following treatment with GLP-2 or DEX are in striking contrast to the increases in sugar transport seen in older animals treated with these hormones (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998, Thiesen et al., 2002; Thiesen et al., 2003). Although both the glucocorticoid receptor and the GLP-2 receptor are present in suckling animals (Henning et al., 1975; Lovshin et al., 2000), there is a lack of response to these hormones, suggesting that there are age-related changes in the responsiveness to these treatments.

In models of intestinal adaptation, it is usually the Vmax which is responsible for alterations in nutrient absorption (Ferraris and Diamond, 1997). In this study, the treatments had variable effects on the Km, depending upon the method used to estimate the value. The Km in the jejunum was increased by GLP-2+DEX, while the effect of GLP-2 or DEX alone was variable depending on the plot used (Table 19). Thus, we cannot conclude with confidence what is the effect of these treatments in the value of the Km when DEX or GLP-2 given to suckling animals. More consistent data was obtained from the ileum, where decreases in the Km were seen with DEX, GLP-2 and GLP-2+DEX.

The changes in the Vmax seen in this study could be due to alterations in the number of sodium dependent glucose transporters (SGLT1) in the BBM, or due to changes in the intrinsic activity of the existing transporters. The phenomenon of altering the intrinsic activity of a transporter, in the absence of changes in protein abundance, has been observed in several studies (Maenz and Cheesman, 1986; Corpe et al., 1996; Kellett and Helliwell, 2000). Post-translational modifications, such as phosphorylation or interactions with regulatory proteins may influence the activity of a transporter, as well as the affinity for its substrate, a as reflected by changes in the Km. As we did not measure the abundance of SGLT1 in this study, we can only speculate on what may have been the basis for the altered kinetics.

The recruitment of GLUT2 to the BBM has been reported under conditions where animals were given glucose loads or fed high glucose meals (Helliwell et al., 2000a; Helliwell et al., 2000b; Gouyon et al., 2003). Although GLP-2 has also been shown to stimulate the recruitment of GLUT2 to the BBM (Au et al., 2002), the changes observed in this study were specific to glucose, as fructose uptake was unchanged. This suggests that GLUT2, which transports both fructose and glucose, is unlikely to be responsible for the changes in the Vmax of glucose uptake observed in this study.

DEX increases apoptosis (Foligne et al., 2001) and GLP-2 increases proliferation and decreases apoptosis in the intestine (Drucker et al., 1996; Burrin et al., 2000; Burrin et al., 2005). The increase in crypt depth in the jejunum of suckling animals given DEX, GLP-2 or DEX + GLP-2 (Table 16) may relate to alterations in proliferation or apoptosis, although this was not tested in this study. The increase in villous height and width in the jejunum of animals given DEX (Table 16) is not associated with an alteration in glucose uptake (Table 4). Similarly, despite morphological changes seen in the ileum of animals treated with DEX+GLP-2 (Table 1), fructose uptake was unchanged (Table 18). Again, this demonstrates a disassociation between alterations in nutrient uptake and intestinal morphology.

There are examples in the literature of a late effect of early treatment, such as dietary manipulation in sucklings resulting in a later effect being observed in weanlings (Perin et al., 1997; Jarocka-Cyrta et al., 1998; Perin et al., 1999). With the lack of effect of GLP-2 and DEX+GLP-2 on the Vmax for glucose uptake in sucklings, we were not surprised that the kinetic analysis did not reveal any change in glucose uptake in the 42-day old weanlings, which had been exposed four weeks previously to these treatment during their suckling period. However, DEX did have a late effect on ileal glucose uptake in this study. In contrast to the reductions in the value of Vmax in seen in the ileum of sucklings treated with DEX (Table 19), weanling animals showed increases in glucose uptake in response to DEX (Table 20). This finding suggests that exposure to DEX can have variable effects on intestinal glucose uptake throughout the course of development of the animal. This finding highlights the importance of the early post-natal environment on the development of subsequent physiological functions, and supports our hypothesis that early exposure to DEX has late effects on intestinal sugar transport.

In adult animals, GLP-2 has a trophic effect on the intestine (Drucker et al., 1996). In this study, GLP-2 given to sucklings did not affect villous morphology. Although the lack of effect could be related to the dose or timing of GLP-2 administration, it is also possible that the GLP-2/GLP-2R axis was not fully functional in the young animals used in this study. Exogenous administration of steroids causes precocious maturation of the intestine (Moog et al., 1971; Galand, 1989; Solomon et al., 2001). In this study, a combination of GLP-2+DEX did result in an increase in villous width and crypt depth (Table 16). It is possible that DEX had a precocious effect on the development of the responsiveness of the intestine to the trophic effects of GLP-2, possibly by enhancing the expression and/or activity of the GLP-2 receptor. Furthermore, an additive effect between DEX and GLP-2 may have occurred, as glucocorticoids are known to have a permissive effect on hormones, such as GLP-2, which act on G-protein coupled receptors and increase adenylate cyclase (Michel et al., 1994; Meier, 1997).

In conclusion, dexamethasone (DEX) may have a late effect on increasing sugar uptake in weanling animals. This finding highlights the importance of the early post-natal environment on the development of subsequent physiological functions. In addition, a combination of GLP-2+DEX stimulated increases in intestinal morphology and glucose uptake in suckling animals. Further research is required to determine the relevance of these findings to clinical situations in which it may be beneficial to stimulate intestinal transport in infants with compromised intestinal function.

ACKNOWLEDGEMENTS

We would like to acknowledge the technical assistance of Elizabeth Wierzbicki.

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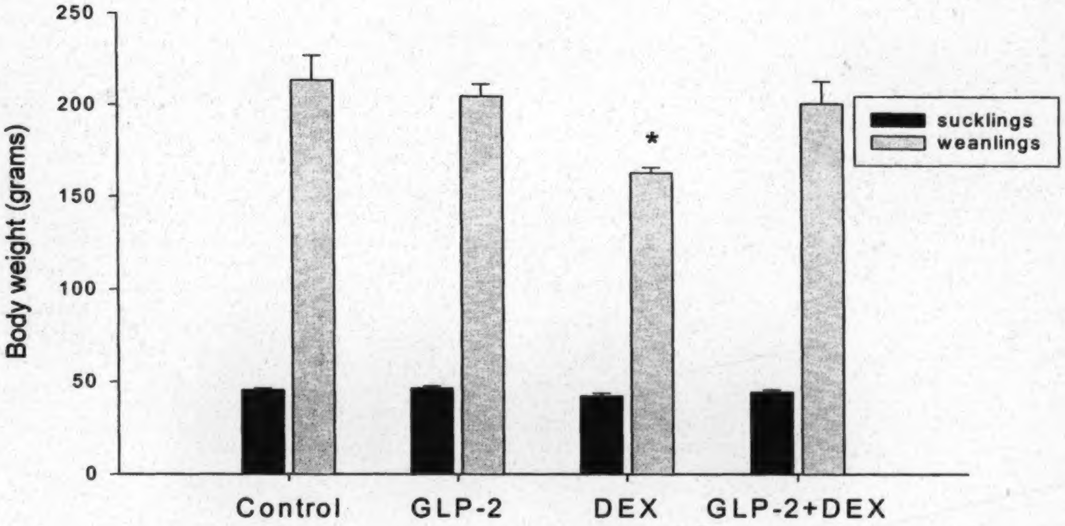

Figure 49. Body weights of suckling and weanling rats injected for 10 days during lactation with GLP-2, DEX, GLP-2 + DEX, or Placebo

Values are expressed mean ± SEM, n=8,

* =$p<0.05$ vs. control

The treatments include GLP-2 (0.1 µg / g twice a day), DEX (0.128 µg / g once a day), and GLP-2 + DEX at those doses, given to suckling animals in the last for 10 days of the lactation. The sucklings were sacrificed on day 21 and the weanlings were sacrificed 28 days after the weaning.

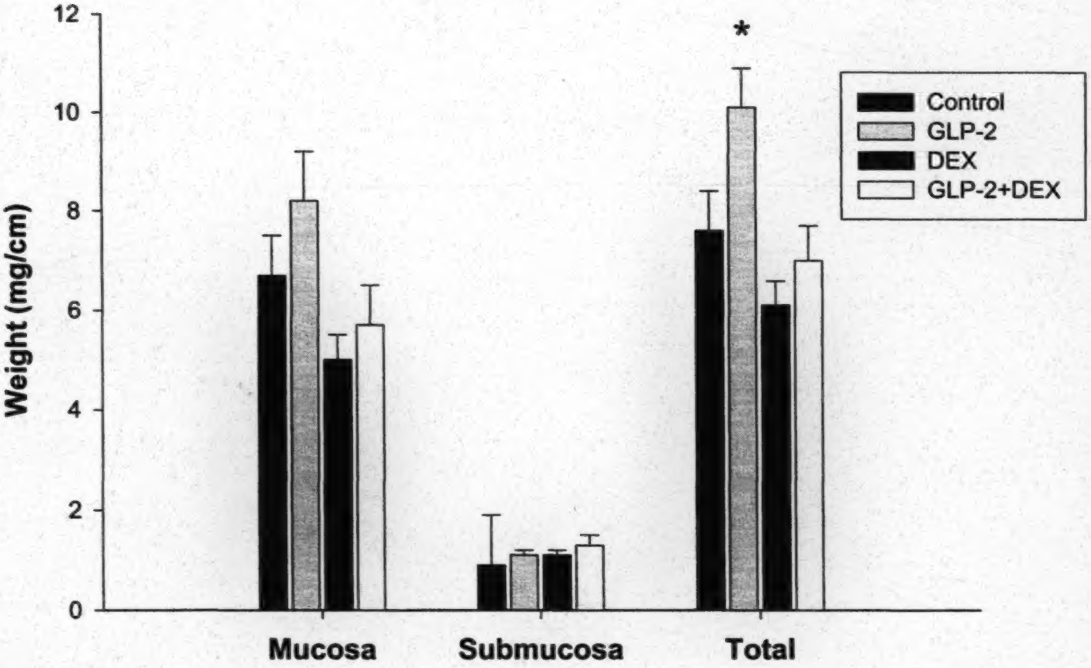

Figure 50. Ileal characteristics of weanling rats injected 10 days during lactation with GLP-2, DEX, GLP-2 + DEX, or Placebo

Values are expressed mean ± SEM, n=8,

*= $p< 0.05$ vs. control

The treatments include GLP-2 (0.1 µg / g twice a day), DEX (0.128 µg / g once a day), and GLP-2 + DEX at those doses, given for 10 days during lactation. The weanling animals were sacrificed on day 49.

Table 16. The effect of treatment of suckling rats with GLP-2, DEX and GLP-2 + DEX on jejunal and ileal morphology of the suckling offspring

		CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	Villous height	352.7±12.0	300.8±8.7	486.4±5.6*	406.2±39.1
	Villous width (base)	75.0±5.1	72.9±2.9	105.4±1.0*	105.0±4.8*
	Villous width (mid)	68.8±5.0	75.7±0.1	61.1±2.6	76.4±9.7
	Crypt depth	53.9±5.7	66.1±3.7*	70.5±0.5*	74.0±1.6*
	Distance/5 villi	39.0±6.0	25.7±2.3	31.3±4.2	29.1±1.2
	Distance/5 cells	46.1±0.7	44.4±0.7	31.0±2.8*	40.1±4.2
ILEUM	Villous height	236.9±33.5	175.2±18.1	261.3±59.5	443.8±14.1*
	Villous width (base)	72.5±10.4	62.4±2.1	86.7±7.8	160.7±11.7*
	Villous width (mid)	72.0±6.8	62.5±2.5	62.2±5.6*	126.6±12.0*
	Crypt depth	49.8±4.8	52.3±4.8	69.7±5.8	124.2±13.6*
	Distance/5 villi	455.8±34.4	348.2±15.7*	424.9±29.3	958.3±27.6*
	Distance/5 cells	27.4±5.2	24.9±1.0	23.9±0.8	45.0±1.7*

Values are expressed mean ± SEM, n=8.

*= $p<0.05$ vs. control

The treatments include GLP-2 (0.1 µg / g twice a day), DEX (0.128 µg / g once a day), and GLP-2 + DEX at those doses, given to the suckling rats for 10 days during lactation. The sucklings were sacrificed on day 19-21.

Table 17. The effect of treatment of suckling rats with GLP-2, DEX and GLP-2 + DEX on jejunal and ileal morphology of the weanling offspring

		CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	Villous height	386.4±12.8	383.4±18.0	370.0±28.1	462.7±19.9*
	Villous width (base)	126.5±7.7	115.3±10.0	125.1±3.1	113.2±4.4
	Villous width (mid)	105.3±1.7	109.9±6.3	86.5±7.8	85.4±7.3
	Crypt depth	91.6±3.1	91.1±3.6	91.5±6.9	93.2±6.1
	Distance/5 villi	695.4±65.0	633.4±74.8	556.0±22.3	527.6±18.7
	Distance/5 cells	24.3±1.3	33.2±1.7*	33.4±0.7*	39.0±1.8*
ILEUM	Villous height	203.2±10.7	149.3±37.9	281.4±13.5*	123.8±13.6
	Villous width (base)	104.0±10.4	92.3±24.7	113.0±7.5	45.6±3.8*
	Villous width (mid)	86.9±2.5	76.4±16.7	80.5±5.3	43.5±3.2*
	Crypt depth	89.6±7.3	68.2±7.1	89.6±11.7	38.3±1.8*
	Distance/5 villi	593.9±27.6	460.9±98.3	593.4±24.5	325.8±54.1*
	Distance/5 cells	24.1±1.3	17.9±2.2	39.3±1.3*	13.4±0.6*

Values are expressed mean ± SEM, n=8.

*= $p<0.05$ vs. control

The treatments include GLP-2 (0.1 µg / g twice a day), DEX (0.128 µg / g once a day), and GLP-2 + DEX at those doses, given to the suckling rats for 10 days during lactation. The weanlings were sacrificed on day 49.

Table 18. The effect of treatment of suckling rats with GLP-2, DEX and GLP-2 + DEX on fructose uptake in suckling and weanling offspring

		CONTROL	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sucklings	13.24±0.7	14.73±0.72	12.23±0.87	13.79±0.91
	Weanlings	13.67±1.74	12.02±1.5	9.45±1.26	14.13±1.75
ILEUM	Sucklings	14.97±0.85	17.29±0.8	12.62±0.9	14.02±1.23
	Weanlings	8.11±1.35	9.15±1.78	8.43±0.96	9.24±2.32

Values are slopes expressed as mean ± SEM, n=8.

There were no statistically significant differences

The treatments include GLP-2 (0.1 μg / g twice a day), DEX (0.128 μg / g once a day), and GLP-2 + DEX at those doses, given to the suckling rats for 10 days during lactation. The sucklings were sacrificed on day 21. The weanlings were sacrificed 28 days after weaning.

Table 19. The effect of treatment of suckling rats with GLP-2, DEX and GLP-2 + DEX on the Vmax and Km for glucose uptake in suckling offspring

	Vmax: Sucklings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	1200±154	1647±353	1658±237	2039±222
	Lineweaver-Burke	1009±119	969±81	1066±99	1314±36
	Wolfee	1196±114	1629±66 *	1608±134 *	1916±75 *
	Eadie-Hofstee	1047±99	1125±82	1223±108	1536±40 *
ILEUM	Sigmaplot	2941±575	2574±513	1957±289	2641±479
	Lineweaver-Burke	1701±78	1605±85	1332±138 *	1689±59
	Wolfee	2681±76	1271±109	1949±297 *	2427±100
	Eadie-Hofstee	1963±88	1844±87	1500±174 *	1929±61
	Km:Sucklings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	14.9±5.2	20.1±10.6	21.9±7.5	29.5±6.9
	Lineweaver-Burke	10.0±0.2	5.2±0.1 *	7.7±0.1 *	12.0±1.1 *
	Wolfee	14.8±0.6	19.4±1.4 *	19.6±0.4 *	24.7±1.2 *
	Eadie-Hofstee	10.3±0.5	6.7±0.0 *	9.9±0.2	15.6±1.4 *
ILEUM	Sigmaplot	32.5±13.2	18.2±9.2	16.9±6.5	18.3±8.4
	Lineweaver-Burke	10.0±0.6	5.4±0.1 *	6.3±0.1 *	5.9±0.5 *
	Wolfee	25.4±1.8	18.0±0.5 *	16.3±1.8 *	17.7±0.7 *
	Eadie-Hofstee	12.7±0.7	6.8±0.2 *	7.8±0.2 *	7.4±0.7 *

Values are expressed mean ± SEM, n=8.

*= $p<0.05$ vs. control

The treatments include GLP-2 (0.1 µg / g twice a day), DEX (0.128 µg / g once a day), and GLP-2 + DEX at those doses, given to the suckling rats for 10 days during lactation. The sucklings were sacrificed on day 21.

Table 20. The effect of treatment of suckling rats with GLP-2, DEX and GLP-2 + DEX on the Vmax and Km for glucose uptake in weanling offspring

	Vmax: Weanlings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	816±45	2933±1157	1003±158	1496±347
	Lineweaver-Burke	1590±62	695±58 *	789±66 *	937±38 *
	Wolfee	806±111	2070±17 *	965±10	1511±80 *
	Eadie-Hofstee	720±83	941±45	846±44	1114±68 *
ILEUM	Sigmaplot	1519±357	1895±418	2711±349	1542±188
	Lineweaver-Burke	1033±106	870±43	2083±110*	1368±315
	Wolfee	1325±93	1661±7	4274±528 *	1560±30
	Eadie-Hofstee	914±86	1089±35	1545±134 *	1519±49 *
	Km:Weanlings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	19.9±2.7	93.4±55.0	33.8±10.9	36.2±16.8
	Lineweaver-Burke	14.0±0.5	12.2±1.4	21.9±1.7 *	19.4±2.6 *
	Wolfee	19.2±2.0	53.4±4.1 *	30.7±5.6 *	39.1±3.3 *
	Eadie-Hofstee	15.0±1.0	11.6±0.4	24.0±2.8 *	22.7±2.0 *
ILEUM	Sigmaplot	39.1±17.9	44.8±18.4	144.7±24.9 *	38.3±9.2
	Lineweaver-Burke	13.3±0.1	10.5±0.9	146.1±11.6 *	33.7±19.7
	Wolfee	28.2±1.4	34.2±4.4	255.5±60.8 *	40.1±8.0
	Eadie-Hofstee	12.6±0.04	14.6±1.5	76.6±21.3 *	38.3±9.8

Values are expressed mean ± SEM, n=8.

*= $p<0.05$ vs. control

The treatments include GLP-2 (0.1 μg / g twice a day), DEX (0.128 μg / g once a day), and GLP-2 + DEX at those doses, given to the suckling rats for 10 days during lactation. The weanlings were sacrificed 28 days after weaning.

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9. DEXAMETHASONE AND GLP-2 GIVEN TO LACTATING RAT DAMS INFLUENCE GLUCOSE BUT NOT FRUCTOSE UPTAKE IN SUCKLING AND WEANLING OFFSPRING

9.1. Introduction

During the ontogeny of the gastrointestinal tract, there are age-dependent changes in carbohydrate, lipid and amino acid absorption (Pacha, 2000; Ferraris, 2001). These changes prepare the intestine for the dietary alterations that occur with weaning. These variations in absorption may be due to changes in nutrient transporters, digestive enzymes, and brush border membrane (BBM) permeability (Ferraris et al., 2000; Henning et al., 1994; Buddington and Malo, 2003). Interactions between diverse stimuli and genetic programming contribute to the morphological and functional maturation of the intestine (Henning et al., 1994; Henning, 1994, Clatworthy and Subramanian, 2001; Nanthakumar et al., 2003). These stimuli are induced by hormones, the enteric nervous system, the mucosa and mesenchyme, as well as by luminal factors such as diet and bacterial flora (Pacha, 2000; Nanthakumar et al., 2003; Jensen et al., 2001).

In adult animals, glucagon-like peptide-2 (GLP-2) administration enhances sugar absorption (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998), as well as the absorption of lipids such as triolein (Brubaker et al., 1997). Recently, GLP-2 was shown to be important in initiating and maintaining the small intestinal adaptive response to resection (Martin et al., 2005). It is not known if GLP-2 influences the absorption of sugars in young animals. Also, glucocorticosteroids such as dexamethasone (DEX) increase the intestinal uptake of sugars and lipids in adult rats (Thiesen et al., 2002; Thiesen et al., 2003), but it is not known if DEX, when given to lactating dams, alters sugar uptake in sucklings. Previous studies with rodents have shown that the lipid content of the maternal diet during pregnancy or lactation influences the absorption of nutrients in the offspring (Jarocka-Cyrta et al., 1998; Perin et al., 1999). Some lactating mothers might be given GLP-2 for treatment of the short bowel syndrome (Jeppesen et al., 2001), or be given glucocorticosteroids for medical conditions such as asthma or inflammatory bowel disease. It is not known if the administration of GLP-2 or DEX to the lactating mother will affect the intestinal sugar uptake of the offspring.

Accordingly, this study was undertaken to determine 1) the influence of GLP-2, DEX, and GLP-2 + DEX, when administered to lactating rat dams on the *in vitro* intestinal uptake of sugars in offspring; 2) if these changes in sugar uptake are due to variations in the intestinal morphology or mass; and 3) if GLP-2, DEX, or GLP-2 + DEX given to the lactating dams have a late effect on sugar uptake in the offspring after weaning.

9.2. METHODS

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and by the Council of the American Physiological Society, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Alberta. Eight two week old pregnant Sprague Dawley rats were obtained from BioScience Animal Services, University of Alberta. The dams were randomized into four groups which received treatment with GLP-2, DEX, GLP-2 plus DEX, or placebo. The treatment was initiated after delivery and was continued until the offspring were 19-21 days old. GLP-2 was administrated in a dose of 0.1 μg/g body weight/day given subcutaneously [sc] twice per day at 7:00 am and 7:00 pm. DEX was administrated in a dose of 0.128 μg/g body weight/day sc once per day at 7:00 pm. The regimen used for GLP-2 + DEX group was GLP-2 0.1 μg/g body weight/day sc twice per day at 7:00 am and 7:00 pm plus DEX 0.128 μg/g body weight/day sc once per day at 7:00 pm. The placebo group received 0.9% saline in a volume equal with the volume of GLP-2 administrated daily per rat sc twice per day at 7:00 am and 7:00 pm.

The number of offspring was decreased after delivery to 12 pups, which were housed with their nursing dams. At weaning, eight offspring per group ("sucklings") were sacrificed for the uptake studies, and eight per group were sacrificed for morphology and immunohistochemistry. The remaining post-weaning animals ("weanlings") were sacrificed for uptake studies at 7 weeks of age (Figure 51).

The animals were housed at a temperature of 21°C, and were exposed daily to 12 hours of light and 12 hours of darkness. During the suckling period the offspring received only the dam's milk. The weanlings were housed in pairs and their water and food were supplied *ad libitum*. The dams and the weanlings were fed standard rat chow (PMI # 5001, Nutrition International LLC, Brentwood, MO, USA). The diets were nutritionally adequate, providing for all known essential nutrient requirements. Body weights were recorded at the time of weaning and then weekly for the next four weeks.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labeled probes included glucose (2-64 mM) and fructose (4-64 mM). The labeled and unlabeled probes were supplied by Amersham Biosciences Inc. (Baie d'Urfe, PQ) and Sigma (St. Louis, MO), respectively. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume (Westergaard and Dietschy, 1974).

Tissue preparation

Eight animals per treatment group were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbital, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed, rinsed with cold saline, and divided into two parts. The proximal half of the intestine beginning at the ligament of Treitz was termed the "jejunum", and the distal half was termed the "ileum". A 2 cm piece from each segment was gently scraped with a glass slide. The mucosal scrapings and the remaining wall of the intestine were dried overnight in an oven at 55°C. The percentage of the intestinal wall comprised of mucosa was calculated. The remaining intestine was everted, and cut into small rings of approximately 2-4 mm each. These intestinal rings were immersed in pre-incubation beakers containing Krebs's buffer (pH 7.2) at 37°C, bubbled with oxygen plus bicarbonate (O_2-CO_2, 95:5 by volume), and were allowed to equilibrate for 5 minutes (Perin et al., 1997). Uptake was initiated by the timed transfer of the tissue rings from the pre-incubation buffer to a 5 ml plastic vial containing [^{3}H]-inulin and ^{14}C-labelled sugars in Kreb's buffer bubbled with oxygen plus bicarbonate that had been equilibrated to 37°C in a shaking water bath. The intestinal rings were incubated in the sugar substrates for 5 minutes.

Determination of uptake rates

The rate of uptake of sugars was terminated by pouring the vial contents onto filters on an Amicon vacuum filtration manifold that was maintained under suction, followed by washing the intestinal rings three times with ice-cold saline. The tissue rings were dried overnight at 55°C. The dry weight of the tissue was determined, the samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Westergaard and Dietschy, 1974). The rates of sugar uptake were determined as nmol of substrate absorbed per 100 mg dry weight of the mucosa per minute (Jm, nmol•100 mg mucosal tissue $^{-1}$• min $^{-1}$). The kinetic constants maximal transport rate (Vmax) and apparent Michaelis affinity constant (Km) were calculated by non-linear regression employing Sigma Plot program (Jandel Scientific, San Rafael, California, USA), as well as from three linear transformations of the uptake data including the Lineweaver-Burke plot, the Wolfee plot and the Eadie-Hofstee plot.

Morphological analysis

To determine the morphological characteristics of the intestine, a vertical section was prepared from the jejunum and the ileum. Hematoxylin and eosin-stained slides were prepared from paraffin blocks. Crypt depth, villous height, villous width, villous width at half height, and cell density were measured using the program MetaMorph 5.05r (Universal Imaging Corporation, Downingtown, PA, USA). The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of four animals in each group.

Statistical analyses

The results were expressed as mean ± standard error of the mean. The statistical significance of the differences between the four groups was determined by analysis of variance (ANOVA). Statistical significance was accepted as $p \leq 0.05$.

9.3. RESULTS

Body weights, Intestinal Weights, and Morphology

GLP-2 or DEX reduced the body weight of sucklings when compared to controls, and this decline was prevented by GLP-2+DEX (Figure 52). GLP-2, DEX, or GLP-2+DEX did not affect the body weights of weanlings.

GLP-2 did not alter the intestinal characteristics of sucklings (Figure 53a). In contrast, DEX decreased the total weight of the jejunal wall, the jejunal mucosal weight, and the percentage of the intestinal wall comprised of mucosa. Neither DEX nor GLP-2+DEX had an effect on the ileum. GLP-2 + DEX prevented the marked loss in jejunal mass seen with DEX alone.

GLP-2 increased the weight of the jejunal mucosa in weanlings when compared to placebo (Figure 53b). DEX had no effect on the intestinal characteristics. GLP-2 + DEX markedly increased the weight of the jejunal submucosa, and decreased the percentage of the jejunal wall comprised of mucosa.

In the jejunum of sucklings, GLP-2 increased villous height, width, crypt depth, distance between villi and distance between cells (Table 21). In contrast, GLP-2 had no effect on the ileal morphology in sucklings (data not shown), or on the jejunal or ileal morphology of weanlings (Table 22). DEX increased jejunal villous height, villous width, distance between villi and distance between cells in sucklings (Table 21). In contrast, in weanlings DEX reduced all of these endpoints in the jejunum and ileum (Table 22). GLP-2 + DEX had no effect on jejunal or ileal morphology in sucklings (Table 21), but in weanlings GLP-2 + DEX was associated with a decline in most of the jejunal and ileal morphological parameters (Table 22).

Sugar uptake

Sucklings:

Jejunal fructose uptake was not affected by GLP-2 or DEX, while GLP-2+DEX decreased fructose uptake in the ileum (Table 23). The value of the Vmax for jejunal glucose uptake was not affected by any of the treatments (Table 24). The Vmax in the ileum was increased by GLP-2 (Sigmaplot, Wolfee and Eadie-Hofstee plot), and by GLP-2+DEX (Lineweaver Burke plot) (Table 24). The Km in the jejunum was increased by

DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot), and by GLP-2 and GLP-2+DEX (Lineweaver Burke and Eadie-Hofstee plot) (Table 24). The Km in the ileum was increased by GLP-2 (Wolfee and Eadie-Hofstee plot) and by GLP-2+DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot).

Weanlings

Although jejunal fructose uptake was not affected by the treatments, ileal fructose uptake was reduced by GLP-2+DEX (Table 23). Both GLP-2 and DEX reduced the Vmax for jejunal glucose uptake (Lineweaver Burke and Eadie-Hofstee plot) (Table 25), while GLP-2+DEX increased the value of the Vmax (Wolfee plot). There were no significant effects of the treatments on the Vmax in the ileum. The Km for jejunal glucose uptake was decreased by DEX (Lineweaver Burke, Wolfee and Eadie-Hofstee plot) and increased by GLP-2 and GLP-2+DEX (Wolfee plot) (Table 25). In the ileum, the Km for glucose uptake reduced by DEX (Lineweaver Burke and Wolfee plot).

9.4. DISCUSSION

Preliminary work has demonstrated that the intestine of suckling rats does not modify its absorption of sugars in response to 10 days treatment with DEX, GLP-2 or DEX + GLP-2 (Drozdowski et al., 2004 unpublished observations). These were surprising findings, since dexamethasone produces precocious development of the digestive and transport function of the intestine (Lebenthal et al., 1972; Sangild et al., 1995; Buchmiller et al., 1994; Meneely and Ghishan, 1982; Guo et al., 1995), and GLP-2 given to adults has a trophic effect on the morphology of the intestine as well as stimulates nutrient uptake (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheesemna and O'Neill, 1998; Drucker et al., 1996). Thus, we were uncertain whether the lack of effect of DEX and GLP-2 on sugar uptake was due to the duration of treatment (10 days), or to the timing of the treatment. Modifying the lipid content of the diets of lactating dams results in alterations in the intestinal uptake of sugars and lipids (Jarocka-Cyrta et al., 1998; Perin et al., 1999). We therefore speculated that giving DEX and GLP-2 to the lactating dams, for a longer duration (21 days), might result in alterations in the ontogeny of sugar absorption in the offspring.

Glucocorticosteroids increase intestinal sugar and lipid uptake in adult rats (Thiesen et al., 2002; Thiesen et al., 2003), and result in the precocious development of intestinal digestive and transport function when given to young animals (Henning et al., 1994). In adult animals, GLP-2 enhances the sugar absorption (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheesemna and O'Neill, 1998). While the role of GLP-2 in the development of intestinal transport function is not known, the GLP-2 receptor is present in the fetal gut (Lovshin et al., 2000). The receptor is active, as the fetus responds to GLP-2 treatment by increasing small intestinal weight and length (Lovshin et al., 2000).

The neonatal environment has important effects on intestinal function later in life. Previous studies with rodents have shown that the lipid content of the maternal diet during pregnancy or lactation influences nutrient absorption in the offspring ((Jarocka-Cyrta et al., 1998; Perin et al., 1999). Accordingly, we hypothesized that treating lactating dams with GLP-2 or DEX would influence intestinal sugar transport in their offspring. The treatments DEX, GLP-2 and DEX + GLP-2 had no effect on the value of Vmax for jejunal glucose uptake in suckling animals (Table 24). The Vmax for ileal glucose uptake was increased by GLP-2, when estimated using the Sigmaplot, Eadie-Hofstee and Wolfee plot. This therefore suggests that GLP-2 does have an enhancing effect on the ileal uptake of glucose when the treatment is administered for 21 days to the lactating dam. This supports the hypothesis that GLP-2 stimulates glucose uptake when given for a longer period of time, and at an earlier stage of development.

In most studies of intestinal adaptation, it is the value of the Vmax rather than the Km which responds to experimental manipulation (Ferraris and Diamond, 1997). We were therefore surprised to see increases in the Km for jejunal and ileal glucose uptake with the three treatments, as estimated from curvilinear as well as the linear transformation plots (Table 24). The increase in the Vmax in the ileum with GLP-2 as well as the increase in the Km in the jejunum and ileum with GLP-2, could potentially be explained by alterations in the abundance of SGLT1. Previous studies have not demonstrated alterations in the effective resistance of the unstirred water layer in this setting (Iordache et al., 2004). While the insertion of a second glucose transporter into the BBM with a higher Km value could potentially explain the higher value of the Km seen with this treatment, our studies were not performed following an oral glucose challenge, which is the methodology required to demonstrate trafficking of GLUT2 to the brush border membrane.

The alterations in the Vmax and Km in the ileum of sucklings (Table 24) treated with GLP-2 were not associated with alterations in intestinal morphology (data not shown). Furthermore, the increases in villous height and width seen with DEX and GLP-2 were not associated with an alteration in the Vmax for jejunal glucose uptake. This adds to the suggestion that the kinetic changes in glucose uptake achieved with GLP-2 were not the result of alterations in morphology. Furthermore, GLP-2 increased villous width and crypt depth in the jejunum of sucklings, demonstrating that GLP-2 does have a biological effect on the offspring when it is administered to the lactating dams.

When suckling animals are given DEX, GLP-2 or DEX + GLP-2 for 10 days, there is no effect on fructose uptake (Drozdowski et al., 2004 unpublished observations). Similarly, despite giving these agents at an earlier stage and for a longer duration, there is still no influence on fructose uptake in sucklings. Other authors have demonstrated the relative immaturity of the GLUT5 transport system for fructose at this age (Jiang et al., 2001), and it is likely that this immaturity is the reason why this system was unresponsive to DEX or GLP-2 when administered to the lactating dam.

Four weeks after the animals were exposed to treatment via their lactating dams, the increase in the ileal Vmax for glucose uptake of GLP-2 was lost. Instead, there was a late effect, a decline in the value of Vmax in the jejunum for glucose uptake in animals treated with GLP-2 (Table 25). Morphological changes could not explain these alterations in uptake, suggesting that the effect was specific and not merely a result of changes in intestinal surface area. Furthermore, there was a late effect of earlier administration of DEX, with a decline in value of Vmax and Km in the jejunum in weanlings and a decline in the value of the Km in the ileum. This also demonstrates that prior exposure of the animals to DEX administered to the lactating dams results in a reduction in glucose uptake in later life.

Finally, there is no data on the passage of GLP-2 across the placenta or across the lactating mammary gland. Thus, it is not possible for us to say that the morphological and transport changes seen with GLP-2 are necessarily a direct effect of the GLP-2, rather than indirect effect on other peptides crossing the placenta, or mammary gland into milk. Nonetheless, it should be pointed out that GLP-2 receptors have been described in fetal and neonatal animals (28).

This study demonstrates the importance of the early environment on the programming of intestinal transport. While maternal GLP-2 treatment has the potential to increasing glucose uptake in suckling rats, the late effects on the reduction of glucose uptake may have potentially detrimental effects on the nutritional status of older animals.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Elizabeth Wierzbicki.

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Table 21. The effect of treatment of lactating rat dams with GLP-2, DEX and GLP-2 + DEX on jejunal morphology of the suckling offspring

		CONTROL	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Villous height	353.8±28.4 **a**	666.5±38.3 **c**	544.8±39.8 **bc**	416.7±64.8 **ab**
	Villous width (base)	78.5±7.0 **a**	171.4±4.6 **c**	129.4±4.0 **b**	110.7±19.3 **ab**
	Villous width (mid)	79.1±3.2 **a**	136.6±9.9 **b**	99.8±4.6 **a**	92.4±11.8 **a**
	Crypt depth	58.2±6.1 **a**	140.4±9.8 **b**	83.7±1.8 **a**	72.6±8.8 **a**
	Distance/5 villi	488.6±38.1 **a**	1048.7±42.1 **c**	813.3±43.1 **b**	570.9±98.7 **a**
	Distance/5 cells	29.5±1.1 **a**	42.1±3.0 **b**	46.7±1.0 **b**	33.2±4.0 **a**

Values are mean ± SEM, n=8

Values with different letters are significantly different ($p<0.05$) by ANOVA.

The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during lactation. The sucklings were sacrificed on day 19-21.

Table 22. The effect of treatment of lactating rat dams with GLP-2, DEX and GLP-2 + DEX on jejunal and ileal morphology of the weanling offspring

		CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	Villous height	727.1±44.9 **a**	720.9±77.6 **a**	392.8±44.2 **b**	441.9±15.1 **b**
	Villous width (base)	230.6±3.3 **a**	240.8±7.3 **a**	105.9±7.1 **c**	154.5±16.7 **b**
	Villous width (mid)	200.3±4.8 **a**	187.3±7.2 **a**	82.5±4.1 **c**	117.9±17.3 **b**
	Crypt depth	147.9±5.7 **a**	150.6±11.9 **a**	76.9±3.2 **b**	114.2±14.7 **a**
	Distance/5 villi	1218.4±47.9 **a**	1234.3±42.0 **a**	565.2±21.4 **c**	865.4±108.1 **b**
	Distance/5 cells	46.1±0.7 **a**	44.4±0.7 **a**	31.0±2.8 **b**	40.1±4.2 **a**
ILEUM	Villous height	583.6±27.2 **a**	635.6±27.1 **a**	280.7±8.1 **b**	324.2±15.0 **b**
	Villous width (base)	225.3±20.2 **a**	223.3±19.8 **a**	110.3±6.4 **b**	114.1±8.2 **b**
	Villous width (mid)	148.1±8.9 **a**	159.0±14.3 **a**	77.9±1.5 **b**	79.0±5.9 **b**
	Crypt depth	138.2±9.1 **a**	168.0±3.8 **a**	78.7±4.2 **c**	70.6±5.2 **c**
	Distance/5 villi	1098.4±32.0 **a**	1079.2±22.8 **a**	526.8±21.7 **b**	547.3±19.8 **b**
	Distance/5 cells	85.9±7.3 **a**	69.8±7.0 **a**	28.8±2.5 **b**	35.8±1.3 **b**

Values are expressed mean ± SEM, n=8

Values with different letters are significantly different ($p<0.05$) by ANOVA.

The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during lactation. The sucklings were sacrificed on day 19-21 and the weanlings were sacrificed on day 49.

Table 23. The effect of treatment of lactating rat dams with GLP-2, DEX and GLP-2 + DEX on fructose uptake in suckling and weanling animals

		CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	Suckling	12.97±1.97	14.08±1.52	16.73±3.15	14.66±1.33
	Weanling	15.32±1.28	12.84±4.16	11.54±0.89	9.44±1.07
ILEUM	Suckling	20.36±2.72	16.87±2.45	10.40±2.55	23.23±2.25
	Weanling	12.88±0.83 **a**	9.85±1.17 **ab**	10.8±1.27 **ab**	8.68±0.75 **b**

Values are slopes expressed as mean ± SEM, n=8

Values with different letters are significantly different ($p<0.05$) by ANOVA.

The treatments include GLP-2 (0.1 µg/g twice a day), DEX (0.128 µg/g once a day), and GLP-2 + DEX at those doses, given during lactation. The sucklings were sacrificed on day 19-21, and the weanlings were sacrificed on day 49.

Table 24. The effect of treatment of lactating rat dams with GLP-2, DEX and GLP-2 + DEX on the Vmax and Km for glucose uptake in suckling animals

	Vmax: Sucklings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	2213±298	2160±301	3078±490	2217±254
	Lineweaver-Burke	1783±216	1664±187	2217±312	2151±244
	Wolfee	2463±412	2273±283	3003±313	2433±168
	Eadie-Hofstee	1913±251	1806±209	2368±326	2097±228
ILEUM	Sigmaplot	1275±31 **a**	3223±423 **b**	1501±131 **ab**	2044±622 **ab**
	Lineweaver-Burke	1292±274 **a**	2347±350 **a**	1546±426 **a**	4831±144 **b**
	Wolfee	1233±296 **a**	3300±330 **b**	1477±426 **a**	2188±242 **a**
	Eadie-Hofstee	1282±269 **a**	2602±336 **b**	1472±414 **a**	1270±216 **a**
	Km:Sucklings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	8.4±3.8	11.4±4.7	23.8±8.8	7.8±3.0
	Lineweaver-Burke	3.9±0.1 **a**	5.3±0.4 **b**	11.8±0.8 **d**	7.2±0.2 **c**
	Wolfee	11.7±1.2 **a**	13.1±0.2 **a**	21.9±2.6 **b**	10.9±1.1 **a**
	Eadie-Hofstee	4.7±0.02 **a**	6.3±0.3 **b**	12.7±0.8 **c**	6.4±0.2 **b**
ILEUM	Sigmaplot	1.8±0.3	16.3±5.8	3.6±1.5	27.0±18.3
	Lineweaver-Burke	2.0±0.2 **a**	7.1±0.5 **a**	4.3±0.01 **a**	86.0±21.0 **b**
	Wolfee	1.2±0.8 **a**	16.6±5.2 **b**	3.5±0.2 **ab**	33.0±4.0 **c**
	Eadie-Hofstee	1.8±0.2 **a**	8.8±0.9 **b**	3.4±0.08 **a**	9.6±0.3 **b**

Values are expressed mean ± SEM, n=8, Values with different letters are significantly different ($p<0.05$) by ANOVA.

Vmax units: nmol • 100 mg mucosal tissue $^{-1}$ • min^{-1}; Km units: mM

The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during lactation. The sucklings were sacrificed on day 19-21.

Table 25. The effect of treatment of lactating rat dams with GLP-2, DEX and GLP-2 + DEX on the Vmax and Km for glucose uptake in weanling animals

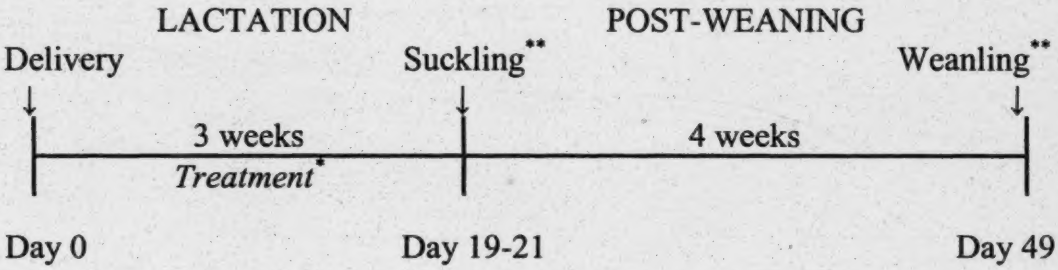
	Vmax: Weanlings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	2381±328	30200±190900	1282±150	3769±1260
	Lineweaver-Burke	1838±78 **a**	745±58 **b**	1017±114 **b**	1570±128 **a**
	Wolfee	2457±118 **b**	1376±73 **a**	1391±135 **a**	3247±302 **c**
	Eadie-Hofstee	1928±66 **a**	908±63 **b**	1092±120 **b**	1858±166 **a**
ILEUM	Sigmaplot	1419±224	1494±315	978±150	1290±202
	Lineweaver-Burke	1172±124	799±17	842±82	1136±136
	Wolfee	1468±141	1401±59	1089±95	1333±205
	Eadie-Hofstee	1191±120	923±63	893±85	1159±157
	Km:Weanlings	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	21.5±7.1	1528±10010	9.2±3.5	58.0±33.3
	Lineweaver-Burke	15.1±0.5 **a**	13.2±1.7 **a**	4.2±0.3 **b**	13.9±0.4 **a**
	Wolfee	24.3±0.5 **a**	38.1±4.7 **c**	11.6±0.0 **b**	44.6±0.2 **d**
	Eadie-Hofstee	14.3±0.9 **a**	17.2±2.2 **a**	5.1±0.3 **b**	15.6±0.2 **a**
ILEUM	Sigmaplot	18.2±7.3	29.9±13.5	6.9±3.8	17.1±6.9
	Lineweaver-Burke	13.8±0.2 **b**	7.6±2.9 **ab**	4.3±0.2 **a**	12.7±0.2 **b**
	Wolfee	20.7±0.1 **a**	24.9±5.0 **a**	10.7±0.1 **b**	18.4±1.4 **a**
	Eadie-Hofstee	12.7±0.1	9.2±5.7	4.8±0.2	12.9±0.6

Values are expressed mean ± SEM, n=8, Values with different letters are significantly different ($p<0.05$) by ANOVA.

Vmax units: nmol • 100 mg mucosal tissue $^{-1}$ • min^{-1}; Km units: mM

The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during lactation. The weanlings were sacrificed on day 49.

Figure 51. Experimental design


LACTATION
POST-WEANING
Delivery
Suckling**
Weanling**
3 weeks
Treatment*
4 weeks
Day 0
Day 19-21
Day 49

* Treatment with GLP-2, DEX, GLP-2 + DEX, and Placebo was administrated each day during lactation.

** Uptake studies were performed at day 19-21 ("suckling") and day 49("weanling").

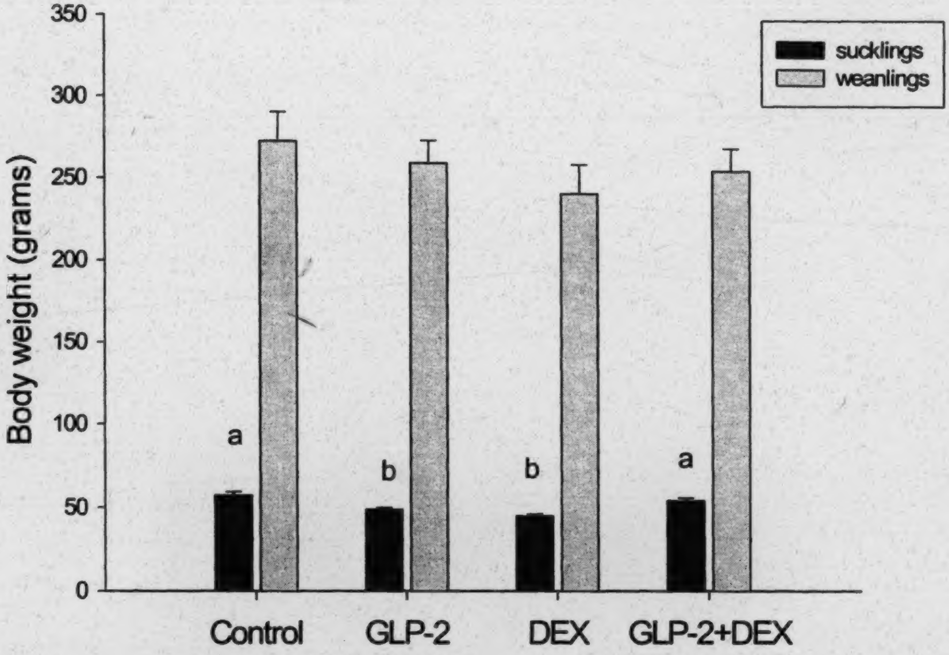


Figure 52. Body weights of suckling and weanling rats of dams injected through lactation with GLP-2, DEX, GLP-2 + DEX, or Placebo

Values are expressed as mean ± SEM, n=8

Values with different letters are significantly different ($p < 0.05$) by ANOVA

The treatments include GLP-2 (0.1 μg / g twice a day), DEX (0.128 μg / g once a day), and GLP-2 + DEX at those doses, given during lactation. The suckling animals were sacrificed on day 19-21, and weanlings on day 49.

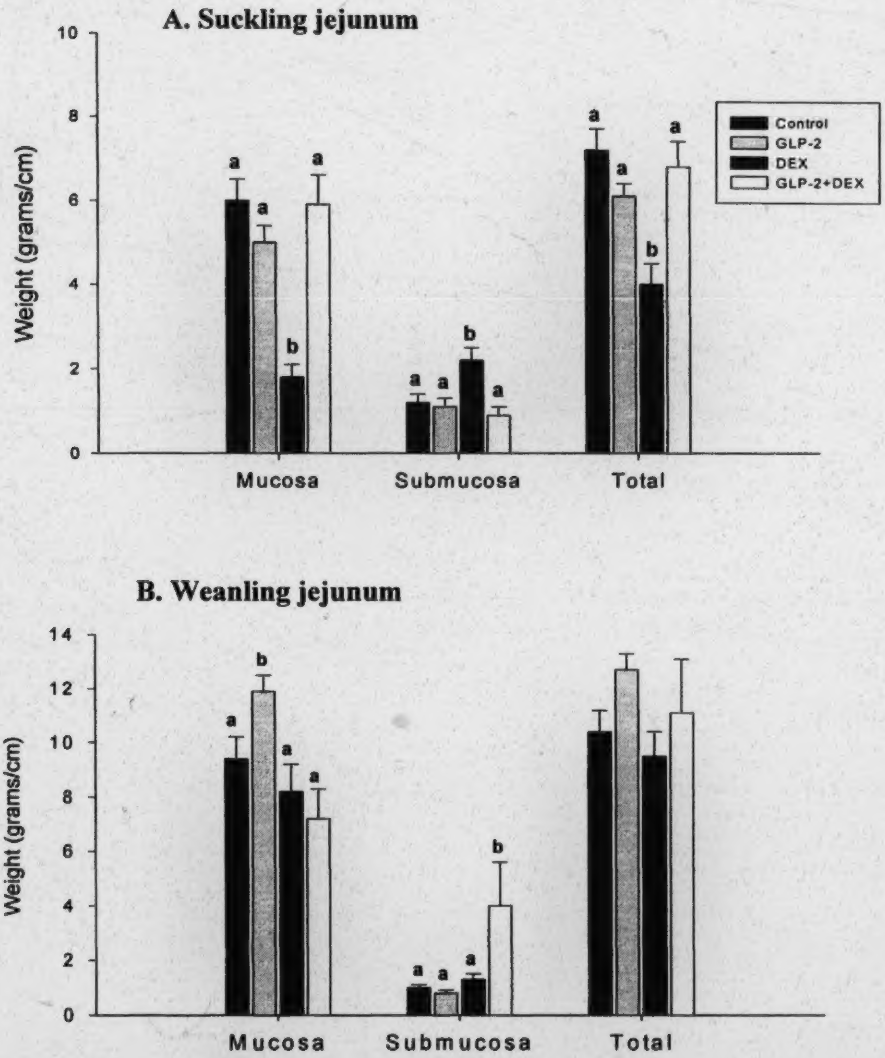


Figure 53. Jejunal characteristics of A) suckling and B) weanling rats of dams injected through lactation with GLP-2, DEX, GLP-2 + DEX, or Placebo

Values are expressed mean ± SEM, n=8

Values with different letters are significantly different ($p < 0.05$) by ANOVA. The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during lactation. The sucklings were sacrificed on day 19-21, and the weanling animals were sacrificed on day 49.

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10. MATERNAL DEXAMETHASONE AND GLP-2 HAVE EARLY EFFECTS ON INTESTINAL SUGAR TRANSPORT IN THEIR OFFSPRING

10.1. Introduction

The ontogeny of the intestinal tract includes all the events involved in the development and maturation of the gut in early life. This complex process involves morphological maturation with the transition from the endodermal tube to the villous-crypt architecture, functional maturation of the digestive and absorptive functions, as well as barrier properties of the mucosa (Henning et al., 1994; Thiesen et al., 2000; Paulsen et al., 2003). The digestive functions exhibit age-dependent alterations in the absorption of nutrients during the suckling and weanling period (Sanderson and Walker, 1994). These variations are due to alterations of the abundance and/or activity of the transporters and digestive enzymes, as well as to changes in the permeability of the brush border membrane (BBM) (Henning et al., 1994; Sanderson and Walker, 1994;; Buddington and Malo, 2003; Nanthakumar et al., 2003).

Glucagon-like peptide-2 (GLP-2) enhances the absorption of sugars in adult animals (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998), but it is not known if GLP-2 influences the intestinal absorption of sugars in young animals. In adult rats, glucocorticosteroids (GC) increase the uptake of both sugars and lipids in young as well as mature animals (Thiesen et al., 2002; Thiesen et al., 2003). While glucocorticosteroids increase apoptosis (Foligne et al., 2001), in contrast GLP-2 increases proliferation and decreases apoptosis in the intestine of adult animals (Drucker et al., 1996; Burrin et al., 2000; Burrin et al., 2005). These changes in the cell cycle may indirectly influence sugar absorption by altering the population of mature, transporting enterocytes.

Data from animal studies show that manipulating the maternal diet has effects on the intestinal uptake of nutrients in the offspring (Perin et al., 1997; Jarocka-Cyrta et al., 1998). This highlights the importance of the fetal and neonatal environment in the health of an infant. GC may be administered to pregnant and lactating mothers, and it is not known if this affects sugar absorption in their offspring, or if the effects of GC can be modified by GLP-2.

A number of intracellular signals and signaling pathways have been implicated in the regulation of intestinal sugar uptake. Intestinal glucose uptake is modulated by both PKC and PKA (Vayro and Silverman, 1999; Helliwell et al., 2000a; Veyhl et al., 2003). PI3K is important in both IGF-1 and EGF stimulated intestinal glucose uptake (Alexander and Carey, 2001; Millar et al., 2002). Furthermore, the MAPK pathway and the PI3K pathway are thought to be important in the PKCβII mediated recruitment of GLUT2 to the intestinal BBM (Helliwell et al., 2000b). Although the effect of DEX on the PI3K pathway is unknown, GLP-2 induced proliferation in the intestine is PI3K dependent (Yusta et al., 1999; Jasleen et al., 2000).

Accordingly, this study was undertaken to determine 1) the influence of GLP-2, dexamethasone (DEX), and GLP-2 + DEX, when administered to pregnant and lactating rat dams, on the intestinal *in vitro* uptake of glucose and fructose in their suckling offspring; and 2) if alterations in the uptake of sugars are associated with variations in intestinal morphology, the abundance of the sugar transporters, or the abundance of selected signals known to regulate sugar transport.

10.2. MATERIALS AND METHODS

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and by the Council of the American Physiological Society, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Alberta. Eight one week old pregnant Sprague Dawley rats were obtained from Bio Science Animal Services, University of Alberta. The dams were randomized into four groups which received treatment with GLP-2, DEX, GLP-2 plus DEX, or placebo. Treatment was started 10 days before delivery, and was continued until the offspring were weaned at 19-21 days of age. DEX was administered in a dose of 0.128 μg/g body weight/day subcutaneously (sc) once per day at 7 pm. GLP-2 was administered in a dose of 0.1 μg/g body weight/day sc twice per day at 7am and 7 pm. The regimen used for DEX + GLP-2 group was DEX 0.128 μg/g body weight/day sc once per day at 7 pm plus GLP-2 0.1 μg/g body weight/day sc twice per day at 7am and 7 pm. The placebo group received 0.9% saline sc in a volume equal to the volume used for GLP-2 administered daily per rat, twice per day at 7 am and 7 pm.

After delivery, the number of offspring was culled to 12 pups, which were housed with their dams. This resulted in 2 dams and 24 pups in each group. At weaning, eight offspring per group ("sucklings") were sacrificed for the uptake studies and eight per group were sacrificed for morphology and immunohistochemistry (Figure 54).

The animals were housed at a temperature of 21°C, and were exposed daily to 12 hours of light and 12 hours of darkness. During the suckling period the offspring received only the dam's milk. Water and food were supplied *ad libitum* to the dams. The dams were fed standard rat chow, PMI # 5001 (Nutrition International LLC, Brentwood, MO, USA). The diets were nutritionally adequate, providing for all known essential nutrient requirements. Body weights of the offspring were recorded at the time of weaning.

Uptake Studies

Probe and marker compounds

The (^{14}C)-labelled probes included glucose (2-64 mM) and fructose (4-64 mM). The labeled and unlabeled probes were supplied by Amersham Biosciences Inc (Baie d'Urfe, PQ) and Sigma (St. Louis, MO), respectively. (^{3}H)-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume (Westergaard and Dietschy, 1974).

Tissue preparation

Eight animals per treatment group were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbital, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with 150 ml cold saline. The intestine was divided into two parts: the proximal half of the intestine beginning at the ligament of Treitz was termed the "jejunum", and the distal half was termed the "ileum". A 2 cm piece of each segment of jejunum and ileum was gently scraped with a glass slide. The mucosal scrapings and the remaining wall of the intestine were dried overnight in an oven at 55°C. The percentage of the intestinal wall comprised of mucosa was calculated. The remaining intestine was everted, and cut into small rings of approximately 2-4 mm each. These intestinal rings were immersed in pre-incubation beakers containing Krebs's buffer (pH 7.2) at 37°C, bubbled with oxygen plus bicarbonate (O_2-CO_2, 95:5 by volume), and were allowed to equilibrate for 5 minutes (Perin et al., 1997). Uptake was initiated by the timed transfer of the tissue rings from the pre-incubation buffer to a 5 ml plastic vial containing (^{3}H)-inulin and ^{14}C-labelled sugars in Krebs buffer bubbled with oxygen plus bicarbonate that had been equilibrated to 37°C in a shaking water bath. The intestinal rings were incubated in the lipid substrates for 5 minutes.

Determination of uptake rates

The rate of uptake was terminated by pouring the vial contents onto filters on an Amicon vacuum filtration manifold that was maintained under suction, followed by washing the intestinal rings three times with ice-cold saline. The tissue rings were placed on a glass slide, and were dried overnight in an oven at a constant temperature of 55°C. The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Westergaard and Dietschy, 1974). The rates of sugar uptake were determined as nmol of substrate absorbed per 100 mg dry weight of the mucosa per minute (Jm, nmol•100 mg mucosal tissue $^{-1}$• min $^{-1}$). Because the relationship between fructose uptake and fructose concentration (4-64 mM) was linear in this study, we reported the slope of this relationship. The relationship between glucose uptake and glucose concentration (2-64 mM) in this study was curvilinear. Therefore, the maximal transport rate (Vmax) and apparent Michaelis affinity constant (Km) were calculated by non-linear regression using the SigmaPlot program (Jandel Scientific, San Rafael, California, USA), as well as from three linear transformations of the uptake data including the Lineweaver-Burke plot, the Wolfee plot and the Eadie-Hofstee plot.

Morphological analysis

In order to determine the morphological characteristics of the intestine, a vertical section was prepared from the jejunum and from the ileum. Hematoxylin and eosin stained slides were prepared from paraffin blocks. Crypt depth, villous height, villous width, villous width at half height, villous density and enterocyte size were measured using the program MetaMorph 5.05r (Universal Imaging Corporation, Downingtown, PA, USA). Villous density was determined by measuring the total width of 5 consecutive villi, and enterocyte size was determined by measuring the total width of 5 consecutive enterocytes along the mid-villous region. The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of four animals in each group.

Immunohistochemistry

Jejunal and ileal tissues were embedded in paraffin, and 4-5 micron sections were mounted on glass slides. The sections were heated and placed immediately in xylene (2 x for 5 min each), followed by absolute ethanol (2 x for 2 min each), and were then rinsed with tap water. The slides were incubated in a hydrogen peroxide/methanol solution, and rinsed with tap water. Then they were rehydrated, and the tissue was encircled on the slides with a hydrophobic slide marker (PAP pen, BioGenex, California). The slides were incubated for 15 min in blocking reagent (20% normal goat serum) followed by 30 minutes incubations with primary antibodies directed against SGLT1, GLUT2, GLUT5, $\alpha 1$ $Na^{+}K^{+}$-ATPase, PCNA, PKA, PKC, phospho-p38, GSK-3, NOS3, phospho-Erk1/2, phospho-Akt1/PKBα and mTOR. All antibodies were obtained from Upstate Biotechnology (Lake Placid, NY) with the exception of anti- SGLT1, anti- GLUT5 (Chemicon, Temecula, CA), anti-GLUT2 (Biogenesis, Poole, England), anti-PCNA, anti-phospho-p38, anti-GSK-3 and anti-NOS3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). All antibodies were diluted 1:50 except for PCNA (1:200), SGLT1 (1:500), GLUT2 (1:500) and GLUT5 (1:250). The slides were incubated in LINK® and LABEL®, and with DAB® solution according to the manufacturers' protocol (BioGenex, San Ramon, California). The slides were then washed, stained in hematoxylin, dehydrated in absolute ethanol, and cleared in xylene. The slides were photographed, and the area labeled with antibody was determined using Metamorph 5.05r. The results were expressed as a ratio of the area which was antibody-positive versus the total area. Statistical analyses were based on a minimum of four villi per animal, and four animals per group.

Apoptosis

Measurements of apoptosis were made based on cell morphology and assessed by a trained pathologist (RREU) blinded to the treatment groups. Apoptotic cells were characterized with slight modifications of observations described by Potten et al. (1997). Apoptotic cells consisted of cells with intensely eosinophilic cytoplasm and nuclear chromatin that was either irregular and condensed or irregular and fragmented. The data is expressed as a crude "apoptotic index" in which the number of apoptotic cells is expressed as a percentage of the total epithelial cell numbers within the villus and the crypt.

Statistical analyses

The results were expressed as mean ± standard error of the mean. The statistical significance of the differences between the four groups was determined by analysis of variance (ANOVA) and the Student Newman-Keuls test. Statistical significance was defined as $p \leq 0.05$.

10.3. RESULTS

Body and Intestinal Weights, and Villous Morphology

There was no significant difference in the rate of body weight gain (grams/day) among the dams in the control, GLP-2, DEX, or GLP-2 + DEX groups (data not shown). Also, there were no differences in the body weights of the suckling rats in these four groups (data not shown).

In suckling rats whose pregnant and lactating dams were given DEX or GLP-2 + DEX, there was increased jejunal mucosal weight, and an increase in the percentage of the jejunal wall comprised of mucosa when compared to controls (data not shown). There were no significant changes in the ileum. Giving GLP-2 to the pregnant and lactating rat dams resulted in larger jejunal enterocytes and higher villi in the ileum of the suckling offspring (Table 26).

Sugar Uptake

Jejunal fructose uptake was significantly reduced by GLP-2, DEX and GLP-2+DEX, as compared with controls (Table 27). Reductions in the value of the Vmax for glucose uptake in the jejunum were seen with DEX and GLP-2+DEX (Table 28). These changes were confirmed using the Lineweaver-Burke, Wolfee and Eadie-Hofstee linear transformation plots. The value of the Km for glucose uptake in the jejunum was also reduced by GLP-2+DEX (Table 28). This finding was also confirmed using the Lineweaver-Burke, Wolfee and Eadie-Hofstee linear transformation plots. No significant changes were seen in the ileal uptake of glucose or fructose.

Immunohistochemistry

Transporters

There was no change in the abundance of SGLT1 in the jejunum or ileum when comparing suckling controls with GLP-2, DEX or GLP-2+DEX (Figure 55A and 55B). GLP-2+DEX increased GLUT2 abundance in the jejunum (Figure 55C). In contrast, all three treatments decreased GLUT2 protein abundance in the ileum when compared to controls (Figure 55D). There was no change in the abundance of GLUT5 in the offspring of animals treated with GLP-2, DEX or GLP-2+DEX (Figures 55E and 55F). The abundance of the α1 Na^+K^+-ATPase was not affected by the treatments (Figures 55G and 55H).

Signals

Both GLP-2 and DEX reduced PCNA abundance in the jejunum and ileum while the combination of GLP-2+DEX increased PCNA abundance in the jejunum (Figure 56A). In the ileum, all treatments reduced PCNA abundance (Figure 56B). The apoptotic index in the intestine was not affected by any of the treatments (data not shown)

There was no change in the abundance of PKA, PKC, MAPK, phospho-p38, GSK-3 or NOS3 in the jejunum or ileum of suckling animals treated with placebo, GLP-2, DEX or GLP-2+DEX (data not shown). Jejunal Akt, but not mTOR, was reduced by all three treatments (Figures 56C and 56E). In the ileum, the abundance of Akt and mTOR was decreased by all three treatments (Figures 56D and 56F).

10.4. DISCUSSION

The intestinal absorption of nutrients transported by carriers is characterized kinetically by the value of the maximal transport rate (Vmax) and the apparent Michaelis constant (Km). A greater Vmax suggests a higher abundance of transporter, or the presence of the same amount of transporter protein but greater transport capabilities (ie. greater intrinsic activity). Alterations in transport may be the result of changes in the microenvironment of lipids in the brush border membrane (BBM) surrounding the transporter (Meddings et al., 1990; Keelan et al., 1991; Meddings, 1998), the insertion of the transporter in the BBM in a position that favors transport (Helliwell et al., 2000a; Kellett and Helliwell, 2000; Helliwell et al., 2000a; Gouyon et al., 2003), the recruitment of transporter in cells lining the mid portion of the villus where transport does not usually occur (Fedorak et al., 1991), or a change in the intrinsic activity of the transporter such as occurs with BBM GLUT2 in response to signaling through the PI3K pathway (Helliwell et al., 2000b).

There are several ways to estimate Vmax, such as linear transformations of the Michaelis-Menten equation, or the use of curve-fitting programs (Thomson and Dietschy, 1977). In this study, we used a curve-fitting program (Sigmaplot) to estimate these parameters. This is a method that uses the Michaelis-Menten equation, assuming that the sugar carrier behaves as if it were an enzyme. The resistance of the unstirred water layer, however, may lead to errors in the estimation of the kinetic constants using this method. We have stirred the solutions used in the uptake studies in order to reduce the effective resistance of the unstirred water layer. Still, the use of this plot remains controversial (Meddings and Westergaard, 1989; Fingerote et al., 1994). However, these investigators agree that non-linear methods of analysis generally provide the best approach to estimating kinetic parameters, since they incorporate variable weighting of the data (Meddings and Westergaard, 1989; Fingerote et al., 1994).

We performed three linear transformations of the data to confirm the results as each method of estimation may over- or under-estimate the value of Vmax or Km (Thomson, 1979a; Thomson, 1979b; Thomson, 1981). The Lineweaver Burke plot is commonly used to estimate kinetic parameters. However, this plot places special emphasis on the velocities obtained at lower concentrations, which are often the least reliable. In some cases this has been shown to result in overestimations of both Vmax and Km (Thomson and Dietschy, 1980). The Eadie-Hofstee plot also has limitations

(Thomson, 1979), as all points tend to concentrate near one of the axes.

Decreases in jejunal fructose and glucose uptake were seen in sucklings exposed to GLP-2, DEX, and GLP-2+DEX (Tables 27 and 28). For glucose, the kinetic basis for the reduction seen with GLP-2+DEX was a decline in the value of both the Vmax and the Km. This reduction could not be explained by decreases in body weight, intestinal characteristics or morphology. In fact, there were increases in jejunal mucosal weight and the size of jejunal enterocytes (Table 26). This highlights the complex relationship between intestinal morphology and intestinal transport, and stresses that alterations in intestinal morphology are not necessarily reflected by changes in absorption.

Changes in intestinal sugar uptake are usually due to alterations in the value of the Vmax rather than the Km (Diamond et al., 1984; Ferraris and Diamond, 1989). However, in this study both the Vmax and the Km for glucose uptake were reduced by GLP-2+DEX. Post-translational modifications of the existing transporters may have occurred, resulting in an increase in affinity (decreased value of Km). Alternatively, there may have been a change in the relative abundance of the various sugar transporters in the brush border membrane (BBM). For example, glucose uptake across the BBM is mediated by SGLT1 (low Km), but under some experimental conditions, such as luminal glucose loading or high sugar diets, GLUT2 (high Km) may traffic to the BBM and contribute to glucose and fructose uptake (Helliwell et al., 2000a; Kellett and Helliwell, 2000; Helliwell et al., 2000b; Gouyon et al., 2003). It is unlikely that this was the explanation for the effect of GLP-2 and DEX on the value of the Km, for if GLUT2 had contributed to BBM glucose uptake we would have observed an increase rather than a decrease in the Km.

Both glucose and fructose uptake were affected in a similar fashion by GLP-2, DEX, and GLP-2+DEX (Tables 27-28). The changes in sugar uptake observed were also similar to the changes in lipid uptake observed in a parallel study (Iordache et al., 2004 unpublished data). This suggests firstly that the effects of the treatments were not specific to either the glucose (SGLT1) or the fructose transporter (GLUT5). Secondly, it appears GLP-2 and DEX may have had a generalized effect on nutrient uptake. One may speculate that the treatments may be influencing the properties of the BBM, which would indiscriminately influence the uptake of sugars and lipids, as well as potentially other nutrients.

Fructose and glucose uptake decreased in the suckling animals after treatment of their dams with DEX or GLP2+DEX (Tables 27 and 28). The mechanism of this effect is unknown, but may have important nutritional implications. Data from animal studies show that manipulating the maternal diet has effects on the intestinal uptake of nutrients in the offspring (Perin et al., 1997; Jarocka-Cyrta et al., 1998). This highlights the importance of the fetal and neonatal environment in the health of an infant. This also has potentially important implications for the health of infants whose pregnant or lactating mothers may have taken steroids or GLP-2 to treat medical conditions such as asthma, rheumatoid arthritis, inflammatory bowel disease or short bowel syndrome. Clearly, caution must be exercised in treating mothers with any agent that could alter the development of the offspring's intestine.

GLP-2 is trophic to the mature intestine (Drucker et al., 1996). The GLP-2 receptor is present in the fetal and neonatal gut, and neonatal rats respond to GLP-2 (Lovshin et al., 2000). In our study, the maternal administration of GLP-2 did result in an increase in the jejunal enterocyte size and ileal villous height (data not shown). Thus, GLP2 given to pregnant and lactating rat dams does have a modest enhancing influence an intestinal morphology in sucklings.

We reasoned that if decreased PCNA represented a decline in crypt proliferation, there might be greater sugar uptake due to an increase in the transport maturity of the enterocytes along the crypt-villous axis. While there were modest changes in intestinal morphology with GLP-2, it was surprising that the abundance of PCNA increased only with GLP-2+DEX (Figures 56A and 56B). This suggests that the treatment-associated alterations in morphology were not entirely explained by changes in proliferation, as measured by PCNA. Similarly, the changes observed in uptake and morphology were not explained by changes in programmed cell death, as intestinal apoptosis was not affected by the treatments (data not shown).

The decreases in proliferation (Figures 56A and 56B) observed in response to DEX and GLP-2 may be due to the decreases in Akt and mTOR (Figures 56C-56F). The

PI3K/Akt pathway is thought to transduce proliferative signals from growth factor receptors to the cell cycle machinery of intestinal epithelial cells. Indeed, Akt increases cyclin D expression, promoting entry into the S phase (Sheng et al., 2003), while mTOR regulates several cell cycle proteins including pRb and $p27^{KIP1}$ (Asnaghi et al., 2004). Our data supports the view that Akt/mTOR modulates proliferation in intestinal epithelial cells. While we expected that DEX would reduce intestinal proliferation (Foligne et al., 2001), it was surprising that GLP-2 had a similar effect. In adult animals, GLP-2 increases intestinal proliferation (Drucker et al., 1996; Burrin et al., 2000; Burrin et al., 2005). The immaturity of our animals, or the indirect administration of GLP-2 via the dams, may be responsible for the unexpected effect of GLP-2 on intestinal proliferation. Curiously, the combination of GLP-2 and DEX resulted in the increase in proliferation anticipated with GLP-2 alone. The mechanism of the interaction between GLP-2 and DEX leading to this increase in proliferation warrants further investigation.

While DEX can pass through the placenta and is present in milk (Grosvenor et al., 1993), it is not known if the same is true for GLP-2. Several other growth factors and hormones are either produced by the placenta, or have receptors on the placenta such as IGF-1, IGF-II, and EGF (Shen et al., 1986; Maly and Luthi, 1986; Hofmann et al., 1991; Duello et al., 1994; Fant, 1996). Similarly, many hormones and growth factors are present in breast milk (Koldovsky and Goldman, 1998). Therefore, maternal GLP-2 may directly influence the offspring, or may influence other growth factors or hormones that can pass through or from the placenta, or into the breast milk, and thereby indirectly alter intestinal morphology or reduce sugar uptake in the suckling animals. Furthermore, alterations in milk volume or changes in milk composition may have occurred in response to GLP-2 and DEX, although this was not investigated in this study.

Immunohistochemistry (IHC) was used to assess the protein abundance of sugar transporters (SGLT1, GLUT2, GLUT5) and selected signals of adaptation (PCNA, PKA, PKC, p38, GSK-3, NOS3, Erk1/2, Akt and mTOR). A correlation between IHC staining and protein levels determined using other methods such as Western blotting (Venter et al., 1987; Podhajsky et al., 1997; Dias et al., 2000) or immunoassays (Aasmunstad et al., 1992; Lehr et al., 1997; Bhatnagar et al., 1999; Simone et al., 2000) has been shown. IHC has been used previously in our lab to demonstrate alterations in protein abundance in the intestine, such as with aging or a result of modifications in dietary lipids (Drozdowski et al., 2003a; Drozdowski et al., 2003b; Woudstra et al., 2004). Although the treatments (GLP-2, DEX and GLP-2+DEX) were associated with a decline in the jejunal uptake of glucose and fructose (Tables 27 and 28), there was no reduction in the abundance of the glucose and fructose transporters (SGLT1, GLUT2, GLUT5) (Figures 55A-55F) or Na^+K^+-ATPase (Figures 55G and 55H). In fact, GLUT2 in jejunum was increased with GLP-2+DEX, indicating that the IHC method used was sensitive enough to detect changes in protein abundance. Although the protein level was increased, both glucose and fructose uptake was reduced, highlighting the complex relationship between protein abundance and intestinal function. We did not perform confocal microscopy in this study, so we do not know the exact location of the protein. In light of the reduction in sugar uptake, we speculate that the GLUT2 is largely intracellular and, therefore, non-functional.

GLUT2 is present in the BLM and functions to transport glucose and fructose out of the enterocytes (Thorens et al., 1988; Burant and Bell, 1992; Cheeseman, 1993), and under conditions of sugar loading GLUT2 may traffic to the BBM to augment sugar uptake (Helliwell et al., 2000a; Helliwell et al., 2000b; Kellett and Helliwell, 2000; Gouyon et al., 2003). In these studies, IHC did not distinguish between the BBM or the BLM localization of GLUT2. The decline in sugar uptake in the jejunum of sucklings was not associated with a decline in the abundance of GLUT2 (Tables 27 and 28, Figures 55C and 55D).

The decline in GLUT2 in the ileum with GLP-2, DEX and GLP-2+DEX did not result in a change in sugar uptake (Tables 27 and 28). Thus, the GLUT2 was either in a location where it could not affect sugar uptake, or it was not functionally active. Similarly, ileal PCNA, Akt and mTOR were reduced in the absence of significant changes in ileal uptake. This suggests that ileal sugar uptake may be regulated by other factors, or that the ileum may be less responsive than the jejunum to alterations in these proteins.

Indeed, there has been a long history of reports of discrepancies between glucose uptake and the protein abundance of glucose transporters both in skeletal muscle (reviewed in Furtado et al., 2002), adipose (Barros et al., 1997) and in the intestine (Maenz and Cheeseman, 1986; Corpe et al., 1996; Au et al., 2002; Thiesen et al., 2003; Helliwell et al., 2000b; Drozdowski et al., 2003a; Drozdowski et al., 2003b). Changes in the intrinsic activity of sugar transporters have been observed with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000), and following the activation of MAPK and PI3K (Helliwell et al., 2000b). The post-translational mechanism by which the intrinsic activity of intestinal sugar transporters is regulated is not known, but may involve phosphorylation of the transporter (Hirsch et al., 1996; Ishikawa et al., 1997) or the activation or inhibition of the transporter by a regulatory protein (Veyhl et al., 1993;Veyhl et al., 2003).

The signals measured in this study were selected from physiological literature suggesting that these proteins may be involved in the regulation of intestinal sugar uptake by GLP-2 or DEX (Burrin et al., 2000; Vayro and Silverman, 1999; Helliwell et al., 2000a; Veyhl et al., 2003; Helliwell et al., 2000b; Hirsch et al., 1996; Ishikawa et al., 1997; Helliwell et al., 2003; Cui et al., 2004). For example, it has been reported that PKA activation enhances both intestinal glucose (Hirsch et al., 1996) and fructose transport (Cui et al., 2004). There are conflicting reports of the effect of PKC on glucose transport (Hirsch et al., 1996; Vayro and Silverman, 1999; Millar et al., 2002; Veyhl et al., 2003) with decreases in sugar transport observed with rabbit and rat SGLT1, and increases seen with human SGLT1 expressed in Xenopus Oocytes (Hirsch et al., 1996). PKCβII may be involved in the enhancement of glucose absorption, which is accomplished by the trafficking of GLUT2 to the BBM (Helliwell et al., 2000a; Helliwell et al., 2003), and PI3K may be important in the stimulation of sugar absorption following an oral sugar load (Helliwell et al., 2000b; Helliwell et al., 2003). The MAPK pathway has been implicated in the control of BBM fructose transport, by modulating both the levels and intrinsic activities of GLUT5 and GLUT2 (Helliwell et al., 2000b). GLP-2 has been shown to influence GSK-3 and eNOS (NOS3) in TPN-fed piglets which may indirectly impact intestinal sugar uptake via effects on intestinal blood flow and cell proliferation

(Guan et al., 2003; Burrin et al., 2005). The abundance of PKA, PKC, Erk1/2, p38, GSK-3, and NOS3 were not affected by the treatments in this animal model (data not shown). However, the abundance of Akt and mTOR, members of the PI3K signaling pathway, were affected in this study. PI3K mediates proliferative signals in intestinal epithelial cells. Treating mice with PI3K inhibitors attenuated the intestinal mucosal proliferation associated with oral intake (Sheng et al., 2003). Furthermore, *in vitro* studies show that GLP-2 induced proliferation is mediated by PI3K (Yusta et al., 1999; Jasleen et al., 2000). Interactions between the glucocorticoid receptor and PI3K have been observed in skin (Leis et al., 2004) as well as mast cells (Andrade et al., 2004), supporting the possibility that DEX may influence this pathway in the intestine.

Our data suggests that the intrinsic activity of the transporters was modified, as has been proposed by Kellett and his colleagues (Helliwell et al., 2000b). They showed that the PI3K pathway is involved in the modification of the intrinsic activity of GLUT2 and GLUT5. In this study, all three treatments significantly reduced Akt abundance in the jejunum and ileum of suckling animals (Figures 56C and 56D). Based on Helliwell's results, one would expect a reduction in Akt to produce a reduction in GLUT2 and GLUT5 activity (fructose uptake) coupled with an increase in the abundance of these proteins. Indeed, when GLP-2+DEX were administered, the reduction in Akt seen in the jejunum of sucklings was associated with reduced fructose uptake, while GLUT2 (but not GLUT5) abundance was increased. Therefore, one may speculate that the changes in fructose uptake observed with GLP-2 and DEX are a result of PI3K mediated changes in the intrinsic activity of GLUT2 and GLUT5. To further investigate the role of the PI3K pathway we also determined the effect of treatments on the abundance of mTOR, a downstream member of the PI3K pathway. In general, the changes seen in Akt (Figures 56C and 56D) were mirrored by parallel changes in mTOR (Figures 56E and 56F), further implicating the PI3K pathway.

It is not known if PI3K/Akt modifies the intrinsic activity of SGLT1. However, a study by Alexander and Carey (2001) showed that orogastric IGF-1 treatment increased intestinal glucose uptake in piglets without increasing SGLT1 abundance, suggesting an effect on intrinsic activity of the transporter. Inhibiting Akt blocked the increase in glucose uptake, possibly by modifying the activity of the transporter. We speculate that a similar mechanism may provide an explanation for the changes in glucose uptake observed in this study in response to DEX+GLP2.

PI3K has been implicated in the regulation of GLUT4 trafficking to the plasma membrane in adipocytes or muscle (reviewed in Furtado et al., 2002). Edinger and Thompson (2002) have shown that because Akt mediated cell survival depends on glucose metabolism, Akt is involved in maintaining glucose transporters on the surface of prolymphoid progenitor cells through an mTOR dependent mechanism. Although we did not examine trafficking in our study, it is possible that the activation of the PI3K pathway is associated with an increase in the trafficking of the intestinal sugar transporters to the BBM. Indeed, the recent work of Cui et al. (2005) demonstrated that the PI3-kinase/Akt signaling pathway may be involved in the synthesis and/or BBM recruitment of GLUT5 by luminal fructose in the small intestine of weaning rats. Using immunohistochemistry we would not necessarily detect this type of change, as we have only determined the total amount of protein in the cell. Despite this possibility, several studies have demonstrated that the trafficking of transporter protein to the BBM cannot fully explain changes in intestinal sugar uptake seen after IGF-1, GLP-2 or glucose administration (Alexander and Carey, 2001; Au et al., 2002; Khoursandi et al., 2004). Nevertheless, we appreciate that both alterations in trafficking and intrinsic activity may contribute to the changes seen in sugar uptake. Further work is required to further characterize the relative contributions of each of these mechanisms.

In summary, intestinal sugar uptake is reduced in the offspring of rat dams treated with a combination of GLP-2 and DEX. We speculate that the mechanism responsible for this decrease involves alterations in the intrinsic activity of the sugar transporters, mediated by the PI3K pathway.

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Figure 56. The early effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on the abundance of PCNA, Akt and mTOR protein as determined by immunohistochemistry

Table 26. The effect of treatment of pregnant and lactating rat dams with GLP2, DEX, or GLP2 + DEX on jejunal and ileal morphology of suckling rats

		CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	Villous height	270±35	354±93	279±40	319±20
	Villous width (base)	72±2	83±16	74±12	82±12
	Villous width (mid)	64±3	70±16	66±7	61±8
	Crypt depth	48±4	71±13	56±12	58±4
	Villous density	509±39	675±86	540±29	517±29
	Enterocyte size	30±0.5 a	46±6 b	32±1 a	34±2 a
ILEUM	Villous height	219±10 a	275±15 b	185±19 a	191±15 a
	Villous width (base)	57±3	92±19	69±12	53±5
	Villous width (mid)	58±2	78±12	59±6	47±6
	Crypt depth	33±3	60±12	36±6	32±3
	Villous density	425±16	602±88	517±62	425±17
	Enterocyte size	27±2	31±2	29±2	28±0.4

Values are mean ± sem, n=8

All measurements are in μM (microns)

Comparisons were made between values within a row, values with different letters are significantly different $p<0.05$ by ANOVA.

The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation. The sucklings were sacrificed on day 19-21.

Table 27. The effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on fructose uptake in suckling rats

	CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	32.7±1.8 a	18.5±2.5 b	17.0±2.4 b	13.0±1.2 b
ILEUM	23.3±3.2	19.0±2.8	21.2±5.7	23.7±2.7

Values are mean ± sem, n=8

Comparisons were made between values within a row, values with different letters are significantly different p<0.05 by ANOVA.

The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation.

Table 28. The effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on the Vmax and Km for jejunal glucose uptake in suckling rats

Vmax	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
Sigmaplot	3710±322 a	2910±340 ab	2290±335 b	1892±171 b
Lineweaver-Burke	3521±89 a	2237±405 b	1812±194 b	1812±237 b
Wolfee	3690±83 a	3086±559 ab	2457±358 b	2105±241 b
Eadie-Hofstee	3350±111 a	2417±437 b	1949±227 b	1827±238 b
Km	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
Sigmaplot	21±4 a	11±4 ab	10±5 ab	5±2 b
Lineweaver-Burke	19±4 a	5±0 b	5±0 b	4±0 b
Wolfee	21±4 a	13±0 ab	13±1 ab	8±0 b
Eadie-Hofstee	17±3 a	6±0 b	6±0 b	4±0 b

Values are mean ± sem, n=8

Comparisons were made between values within a row, values with different letters are significantly different $p<0.05$ by ANOVA.

The treatments include GLP-2 (0.1 µg/g twice a day), DEX (0.128 µg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation.

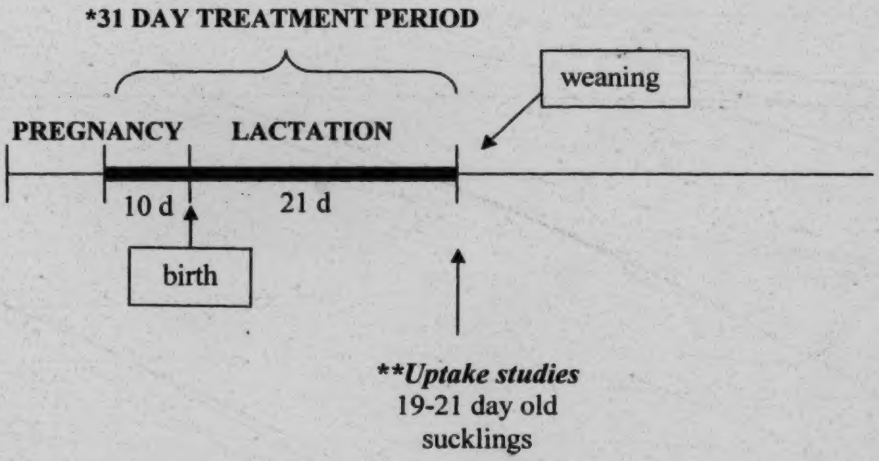
*31 DAY TREATMENT PERIOD
weaning
PREGNANCY
LACTATION
10 d
21 d
birth
**Uptake studies
19-21 day old
sucklings

Figure 54. Experimental Design

*Treatment with GLP-2, DEX, GLP-2 + DEX, and Placebo was administered during the last 10 days of pregnancy and throughout lactation.

**Uptake studies were performed at day 19-21 ("sucklings").

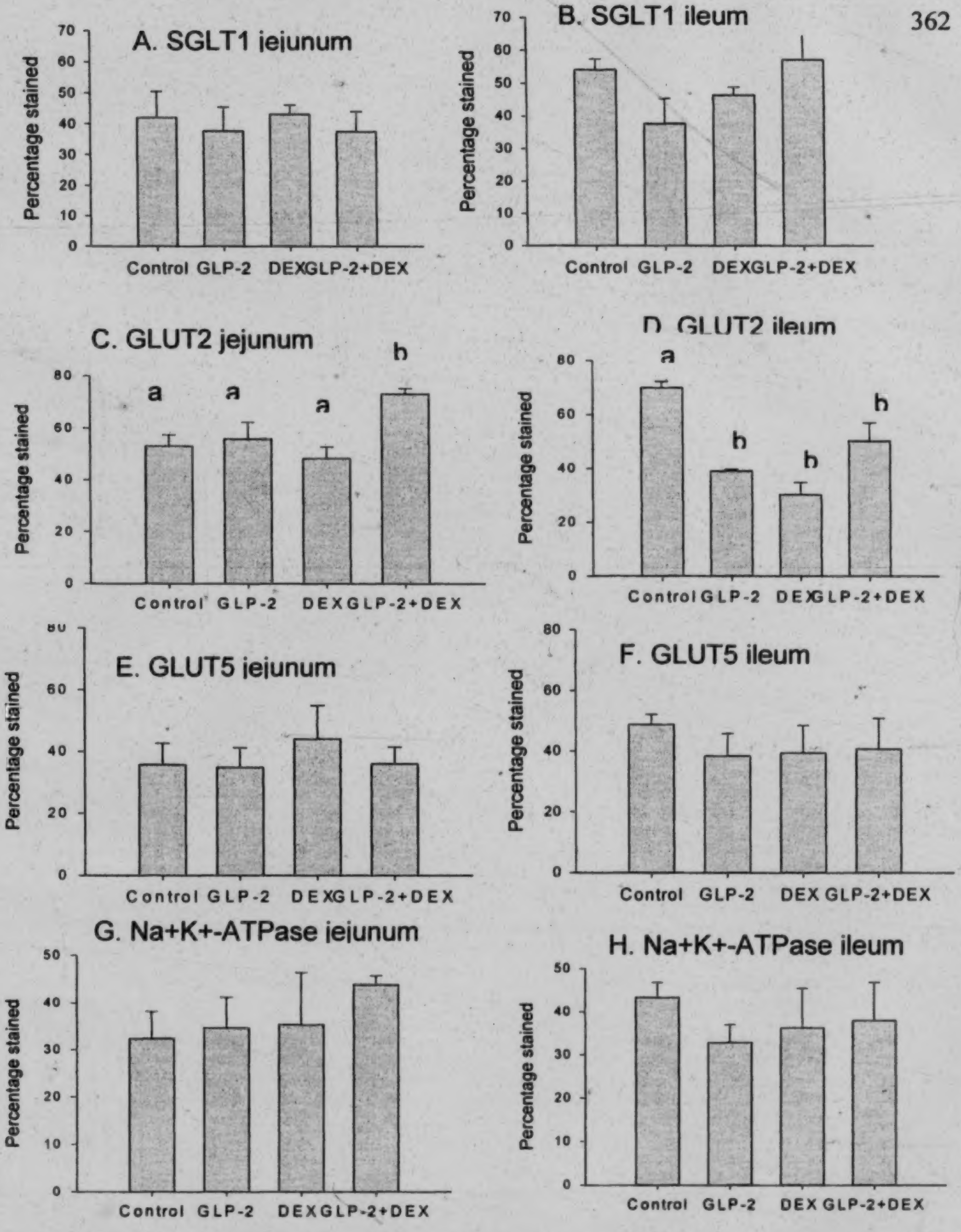

Figure 55. The early effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on the abundance of SGLT1, GLUT2, GLUT5 and $Na^{+}K^{+}$-ATPase protein as determined by immunohistochemistry. Values are mean ± sem, n=4
a, b: values with different letters are significantly different $p<0.05$ by ANOVA.

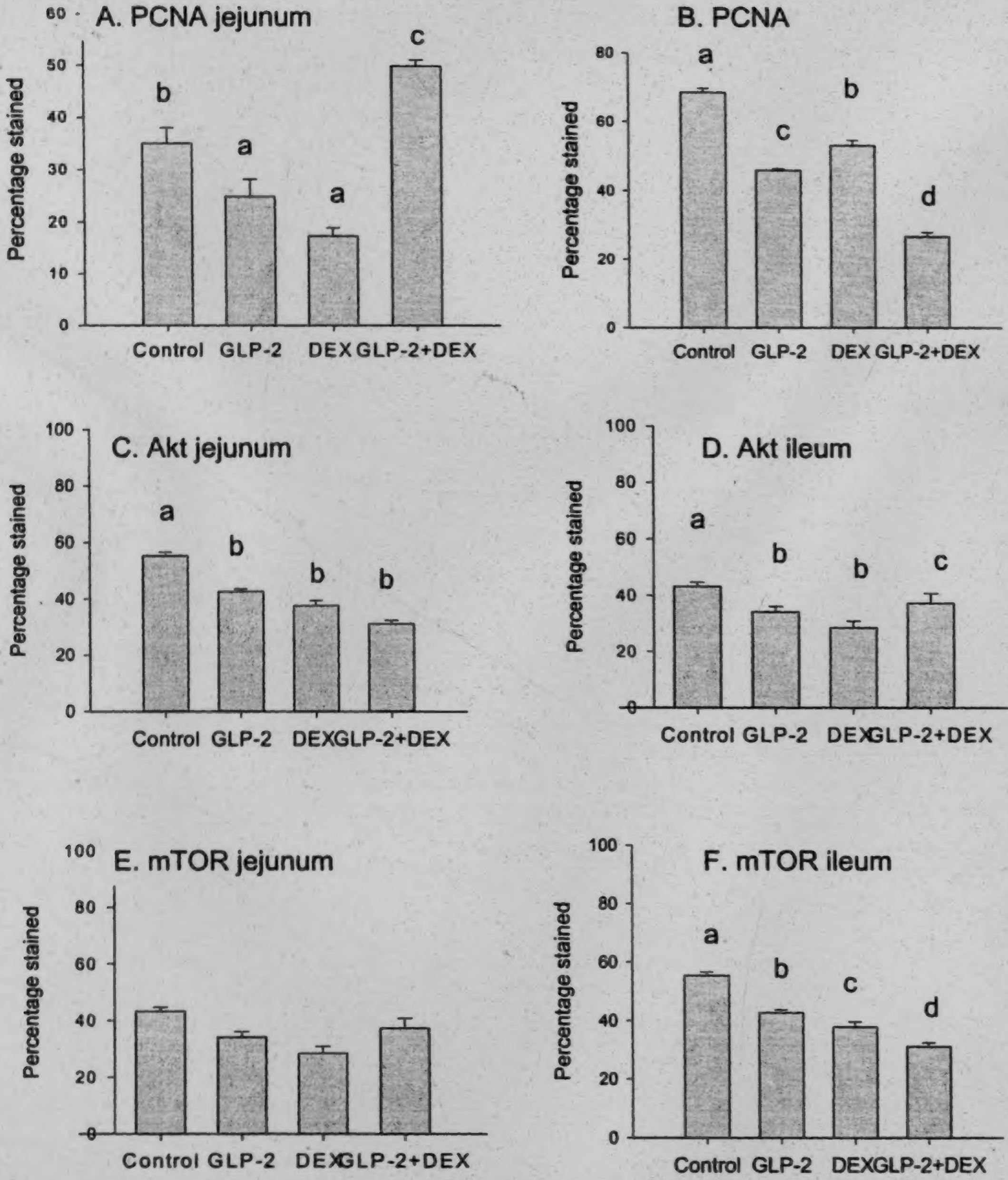

Figure 56. The early effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on the abundance of PCNA, Akt and mTOR protein as determined by immunohistochemistry. Values are mean ± sem, n=4
a, b: values with different letters are significantly different $p<0.05$ by ANOVA.

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11. DEXAMETHASONE AND GLP-2 ADMINISTERED TO RAT DAMS DURING PREGNANCY AND LACTATION HAVE LATE EFFECTS ON INTESTINAL SUGAR TRANSPORT IN THEIR OFFSPRING

11.1. Introduction

"Critical period programming" is a phenomenon by which a biological mechanism is irreversibly turned on or off once during a lifetime in response to prevailing conditions at a critical stage (Karasov et al., 1985). This concept, which has also been referred to as "metabolic programming" or "imprinting" (Lucas, 1998; Lucas, 2000), has been explained using associations between prenatal/neonatal environmental events, alterations in growth and development, and later pathophysiology (Barker et al., 1993; Seckl, 1998).

The ontogeny of the intestine may be influenced by early exposure to nutrients. Several studies have documented changes in intestinal function as a result of alterations in the lipid or carbohydrate content of the weanling diet (Karasov et al., 1985; Thomson and Keelan, 1987; Thomson et al., 1989; Keelan et al., 1990; Perin et al., 1997; Perin et al., 1999). For example, Thomson and Keelan (1987) demonstrated that feeding eight-week old rabbits a low cholesterol diet for two weeks reduced intestinal glucose uptake, and that this effect persisted for at least ten weeks.

Maternal diets also influence intestinal function. For example, feeding diets enriched in saturated fatty acids to lactating dams resulted in increases in sugar uptake in the weanling offspring (Perin et al., 1997). Curiously, these changes were not seen in suckling offspring, suggesting that the mechanisms responsible for adaptation may not be fully developed in these animals. Jarocka-Cyrta et al. (1998) showed that feeding diets differing in fatty acid composition to pregnant and lactating rat dams influenced intestinal function in their offspring. These studies demonstrate that the programming of intestinal function begins very early in life, perhaps even *in utero*.

While early diet clearly influences transport later in life, it is also possible that other factors may influence intestinal function. Several hormones and growth factors have been shown to cause precocious intestinal development, and to potentially stimulate intestinal growth and nutrient transport. However, it is not known if early exposure to these factors has lasting effects on intestinal function.

The enhancing effect of glucagon-like peptide-2 (GLP-2) on intestinal sugar absorption in adult animals is well documented (Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998). However, it is not known if GLP-2 influences the intestinal absorption of sugars in young animals. Similarly, glucocorticosteroids (GC) are known to increase the uptake of both sugars and lipids in mature animals (Thiesen et al., 2002; Thiesen et al., 2003a), but the effects of maternal GC on intestinal function in the offspring have not been studied. Furthermore, glucocorticosteroids increase apoptosis (Foligne et al., 2001), and GLP-2 increases proliferation and decreases apoptosis in the intestine of adult animals (Drucker et al., 1996; Burrin et al., 2000; Burrin et al., 2005). This may indirectly influence sugar absorption by altering the population of mature, transporting enterocytes. GC may be administered to pregnant and lactating mothers, and it is not known if this affects sugar absorption in their offspring, or if any possible effects persist after GC are discontinued. Also, it is not known if the effects of GC can be modified by the co-administration of GLP-2.

The regulation of intestinal sugar uptake is complex and may involve a number of intracellular signals and signaling pathways. *In vitro* studies demonstrate that both PKC and PKA influence intestinal glucose uptake (Vayro and Silverman, 1999; Veyhl et al., 2003). *In vivo* studies using inhibitors of PI3K show that this pathway is important in both IGF-1 and EGF stimulated intestinal glucose uptake (Alexander and Carey, 2001; Millar et al., 2002). The PKCβII mediated recruitment of GLUT2 to the BBM is mediated by the MAPK pathway and the PI3K pathway (Helliwell et al., 2000). Furthermore, although the effect of DEX on the PI3K pathway is unknown, GLP-2 induced proliferation is PI3K dependent (Yusta et al., 1999; Jasleen et al., 2000). Finally, GLP-2 has been shown to influence GSK-3 and eNOS (NOS3) in TPN-fed piglets which may indirectly impact intestinal sugar uptake via effects on intestinal blood flow and cell proliferation (Guan et al. 2003, Burrin et al. 2005).

Accordingly, this study was undertaken to determine 1) the influence of GLP-2, dexamethasone (DEX), and GLP-2 + DEX, when administered to pregnant and lactating rat dams on the intestinal *in vitro* uptake of glucose and fructose in their weanling offspring; and 2) if alterations in the uptake of sugars are due to variations in intestinal morphology, the abundance of the sugar transporters, or the abundance of selected signals known to regulate sugar transport.

11.2. MATERIALS AND METHODS

Animals

The principles for the care and use of laboratory animals, approved by the Canadian Council on Animal Care and by the Council of the American Physiological Society, were observed in the conduct of this study. All experiments were approved by the Animal Ethics Board, University of Alberta. Eight one week old pregnant Sprague Dawley rats were obtained from Bio Science Animal Services, University of Alberta. The dams were randomized into four groups which received treatment with GLP-2, DEX, GLP-2 plus DEX, or placebo. Treatment was started 10 days before delivery, and was continued until the offspring were weaned at 19-21 days of age (Figure 57). DEX was administered in a dose of 0.128 μg/g body weight/day subcutaneously [sc] once per day at 7 pm. GLP-2 was administered in a dose of 0.1 μg/g body weight/day sc twice per day at 7am and 7 pm. The regimen used for DEX + GLP-2 group was DEX 0.128 μg/g body weight/day sc once per day at 7 pm plus GLP-2 0.1 μg/g body weight/day sc twice per day at 7am and 7 pm. The placebo group received 0.9% saline sc in a volume equal to the volume used for GLP-2 administered daily per rat, twice per day at 7 am and 7 pm.

After delivery, the number of offspring was down-sized to 12 pups, which were housed with their dams. This resulted in 2 dams and 24 pups in each group. Post-weaning animals, ("weanlings"), were sacrificed for uptake studies at 7 weeks of age.

The animals were housed at a temperature of 21°C, and each day were exposed to 12 hours of light and 12 hours of darkness. During the suckling period the offspring received only the dam's milk. The weanlings were housed in pairs. Their water and food were supplied *ad libitum*. The dams and the weanlings were fed standard rat chow, PMI # 5001 (Nutrition International LLC, Brentwood, MO, USA). The diets were nutritionally adequate, providing for all known essential nutrient requirements. Body weights were recorded at the time of weaning and then weekly for the next four weeks.

Uptake Studies

Probe and marker compounds

The [^{14}C]-labelled probes included glucose (2-64 mM) and fructose (4-64 mM). The labeled and unlabeled probes were supplied by Amersham Biosciences Inc (Baie d'Urfe, PQ) and Sigma (St. Louis, MO), respectively. [^{3}H]-inulin was used as a non-absorbable marker to correct for the adherent mucosal fluid volume (Westergaard and Dietschy, 1974).

Tissue preparation

Eight animals per treatment group were sacrificed by an intraperitoneal injection of Euthanyl® (sodium pentobarbital, 240 mg/100 g body weight). The whole length of the small intestine was rapidly removed and rinsed with 150 ml cold saline. The intestine was divided into two parts: the proximal half of the intestine beginning at the ligament of Treitz was termed the "jejunum", and the distal half was termed the "ileum". A 2 cm piece of each segment of jejunum and ileum was gently scraped with a glass slide. The mucosal scrapings and the remaining wall of the intestine were dried overnight in an oven at 55°C. The percentage of the intestinal wall comprised of mucosa was calculated. The remaining intestine was everted, and cut into small rings of approximately 2-4 mm each. These intestinal rings were immersed in pre-incubation beakers containing Krebs's buffer (pH 7.2) at 37°C, bubbled with oxygen plus bicarbonate (O_2-CO_2, 95:5 by volume), and were allowed to equilibrate for 5 minutes (Perin et al., 1997). Uptake was initiated by the timed transfer of the tissue rings from the pre-incubation buffer to a 5 ml plastic vial containing [^{3}H]-inulin and ^{14}C-labelled sugars in Krebs buffer bubbled with oxygen plus bicarbonate that had been equilibrated to 37°C in a shaking water bath. The intestinal rings were incubated in the lipid substrates for 5 minutes.

Determination of uptake rates

The rate of uptake was terminated by pouring the vial contents onto filters on an Amicon vacuum filtration manifold that was maintained under suction, followed by washing the intestinal rings three times with ice-cold saline. The tissue rings were placed on a glass slide, and were dried overnight in an oven at a constant temperature of 55°C.

The dry weight of the tissue was determined, and the tissue was transferred to scintillation counting vials. The samples were saponified with 0.75 M NaOH, scintillation fluid was added, and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (Westergaard and Dietschy, 1974). The rates of sugar uptake were determined as nmol of substrate absorbed per 100 mg dry weight of the mucosa per minute (Jm, nmol•100 mg mucosal tissue $^{-1}$• min $^{-1}$). Because the relationship between glucose uptake and concentration was curvilinear, the kinetic constants maximal transport rate (Vmax) and apparent Michaelis affinity constant (Km) were calculated by non-linear regression using the SigmaPlot program (Jandel Scientific, San Rafael, California, USA). In addition, three linear transformations of the uptake data including the Lineweaver-Burk plot, the Wolfee plot and the Eadie-Hofstee plot were performed to confirm these kinetic estimates. Because fructose uptake was linear over the range of concentrations used (4-64 mM), the slopes of the lines were calculated and compared to determine statistically significant differences.

Morphological analysis

In order to determine the morphological characteristics of the intestine, a vertical section was prepared from the jejunum and from the ileum. Hematoxylin and eosin stained slides were prepared from paraffin blocks. Crypt depth, villous height, villous width, villous width at half height, villous density and cell density were measured using the program MetaMorph 5.05r (Universal Imaging Corporation, Downingtown, PA, USA). The group means were obtained based on 10 villi and 20 crypts per slide, with a minimum of four animals in each group.

Immunohistochemistry

Jejunal and ileal tissues were embedded in paraffin, and 4-5 micron sections were mounted on glass slides. The sections were heated and placed immediately in xylene (2 x for 5 min each), followed by absolute ethanol (2 x for 2 min each), and were then rinsed with tap water. The slides were incubated in a hydrogen peroxide/methanol solution, and rinsed with tap water. Then they were rehydrated, and the tissue was encircled on the slides with a hydrophobic slide marker (PAP pen, BioGenex, California). The slides were incubated for 15 min in blocking reagent (20% normal goat serum) followed by 30 minutes incubations with primary antibodies directed against SGLT1, GLUT2, GLUT5, α1 Na^+K^+-ATPase, PCNA, PKA, PKC, phospho-p38, GSK-3, NOS3, phospho-Erk1/2, phospho-Akt1/PKBα and mTOR. All antibodies were obtained from Upstate Biotechnology (Lake Placid, NY) with the exception of anti- SGLT1, anti- GLUT5 (Chemicon, Temecula, CA), anti-GLUT2 (Biogenesis, Poole, England), anti-PCNA, anti-phospho-p38, anti-GSK-3 and anti-NOS3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). All antibodies were diluted 1:50 except for PCNA (1:200), SGLT1 (1:500), GLUT2 (1:500) and GLUT5 (1:250). The slides were incubated in LINK® and LABEL®, and with DAB® solution according to the manufacturers' protocol (BioGenex, San Ramon, California). The slides were then washed, stained in hematoxylin, dehydrated in absolute ethanol, and cleared in xylene. The slides were photographed, and the area labeled with antibody was determined using Metamorph 5.05r. The results were expressed as a ratio of the area which was antibody-positive versus the total area. Statistical analyses were based on a minimum of four villi per animal, and four animals per group.

Apoptosis

Measurements of apoptosis were made based on cell morphology and assessed by a trained pathologist (RREU) blinded to the treatment groups. Apoptotic cells were characterized with slight modifications of observations described by Potten et al. (1997). Apoptotic cells consisted of cells with intensely eosinophilic cytoplasm and nuclear chromatin that was either irregular and condensed or irregular and fragmented. The data is expressed as an "apoptotic index" in which the number of apoptotic cells is expressed as a percentage of the total epithelial cell numbers within the villus and the crypt.

Statistical analyses

The results were expressed as mean ± standard error of the mean. The statistical significance of the differences between the four groups was determined by analysis of variance (ANOVA) and the Student Newman-Kuels test. Statistical significance was accepted for values of $p \leq 0.05$.

11.3. RESULTS

Body and Intestinal Weights, and Villous Morphology

There was no significant difference in the rate of body weight gain (grams/day) among the dams in the control, GLP-2, DEX, or GLP-2 + DEX groups (data not shown). Also, there were no differences in the body weights of the weanling rats in these four groups (data not shown).

In the weanling offspring, there was no effect of GLP-2, DEX, or GLP-2 + DEX on the weight of the jejunal or ileal mucosa, the weight of the intestine, or on the percentage of the intestinal wall comprised of mucosa (data not shown). Similarly, none of the treatments influenced the morphology of the intestine (Table 29).

Sugar Uptake

Jejunal fructose uptake was increased by GLP-2 and GLP-2+DEX, while ileal fructose uptake was reduced by DEX (Table 30). All four of the plots consistently showed that the value of the Vmax for jejunal glucose uptake was increased by DEX+GLP-2 (Table 31). The value of the Vmax in the ileum was also increased by GLP-2+DEX, and this was confirmed using the Wolfee plot (A similar trend was observed with the Lineweaver-Burke and Eadie-Hofstee plot, although the changes were not statistically significant). GLP-2 increased the value of the Vmax as well, although this was only statistically significant when linear transformations (Lineweaver Burke, Wolfee, and Eadie-Hofstee plot) were performed.

The value of the Km in the jejunum was increased by GLP-2+DEX although this was only statistically significant when linear transformations (Lineweaver Burke, Wolfee, and Eadie-Hofstee plot) were performed. The Km in the ileum was increased by GLP-2+DEX, and this finding was confirmed by all three linear transformations (Lineweaver Burke, Wolfee, and Eadie-Hofstee plot). Both GLP-2 and DEX increased the value of the Km in the ileum, although this was only statistically significant when the linear transformations (Lineweaver Burke, Wolfee, and Eadie-Hofstee plot) were performed.

Immunohistochemistry

Transporters

There was no change in the abundance of SGLT1 in the jejunum or ileum when comparing weanling controls with GLP-2, DEX or GLP-2+DEX (Figure 58A and 58B). GLP-2+DEX decreased GLUT2 abundance in the jejunum (Figure 58C and Figure 60), while none of the treatments significantly affected GLUT2 abundance in the ileum (Figure 58D). GLP-2 and DEX decreased GLUT5 protein in the jejunum (Figure 58E), while the treatments had no effect on ileal GLUT5 abundance (Figure 58F). While no changes were observed in jejunal α1 Na^+K^+-ATPase abundance (Figure 58G), each of the three treatments reduced ileal α1 Na^+K^+-ATPase (Figure 58H).

Signals

GLP-2, DEX and GLP-2+DEX decreased apoptosis in the jejunum and ileum of weanlings (Table 32). When the intestinal sections were divided into the upper villus, lower villus and crypt region, similar changes in the number of apoptotic cells were observed in all sections in response to the treatments (data not shown). All of the treatments increased PCNA abundance in the jejunum (Figure 59A), while only DEX increased PCNA in the ileum, with GLP-2 and GLP-2+DEX decreasing the ileal abundance of PCNA (Figure 59B).

There was no change in the jejunal or ileal abundance of PKA, PKC, MAPK, p38, GSK-3 or NOS3 when compared to control animals (data not shown). All three treatments increased Akt abundance in the jejunum (Figure 59C), while GLP-2 and GLP-2+DEX increased Akt, and DEX decreased Akt in the ileum (Figure 59D). All three treatments increased mTOR abundance in both the jejunum and ileum (Figures 59E and 59F).

11.4. DISCUSSION

There are several ways to estimate Vmax, such as linear transformations of the Michaelis-Menten equation, or the use of curve-fitting programs (Thomson and Dietschy, 1977). In this study, we used a curve-fitting program (Sigmaplot) to estimate these parameters. This is a method that uses the Michaelis-Menten equation, assuming that the sugar carrier behaves as if it were an enzyme. The resistance of the unstirred water layer, however, may lead to errors in the estimation of the kinetic constants using this method. We have stirred the solutions used in the uptake studies in order to reduce the effective resistance of the unstirred water layer. Still, the use of this plot remains controversial (Meddings and Westergaard, 1989; Fingerote et al., 1994). However, these investigators agree that non-linear methods of analysis generally provide the best approach to estimating kinetic parameters, since they incorporate variable weighting of the data (Meddings and Westergaard, 1989; Fingerote et al., 1994).

We performed three linear transformations of the data to confirm the results as each method of estimation may over- or under-estimate the value of Vmax or Km (Thomson, 1979a; Thomson, 1979b; Thomson, 1981). The Lineweaver Burke plot is commonly used to estimate kinetic parameters. However, this plot places special emphasis on the velocities obtained at lower concentrations, which are often the least reliable. In some cases this has been shown to result in overestimations of both Vmax and Km (Thomson and Dietschy, 1980). The Eadie-Hofstee plot also has limitations (Thomson, 1979), as all points tend to concentrate near one of the axes.

Changes in intestinal sugar uptake are usually due to alterations in the value of the Vmax rather than the Km (Diamond et al., 1984; Ferraris and Diamond, 1989). However, in this study both the Vmax and the Km for ileal glucose uptake were increased by DEX +GLP-2 (Table 31). Alternatively, there may have been a change in the relative abundance of the various sugar transporters in the brush border membrane (BBM). For example, glucose uptake across the BBM is mediated by SGLT1, but recent evidence suggests that under some experimental conditions, such as luminal glucose loading or high sugar diets, GLUT2 may traffic to the BBM and contribute to the enhanced uptake of both glucose and fructose (Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Gouyon et al., 2003). Because SGLT1 has a low Km (Wright et al., 2003) and GLUT2 has a higher Km (Walmsley et al., 1998) an increase in BBM GLUT2 may explain the increased Km with GLP-2+DEX. However, the animals in this study were not challenged with an oral sugar load, so that enhanced trafficking of GLUT2 to the BBM in this experiment seems unlikely.

The combination of GLP-2+DEX resulted in the most pronounced increase in the value of the Vmax for intestinal glucose uptake, when compared to controls (Table 31). This suggests that the two agents may have a synergistic effect when given in combination. Interestingly, the combination of GLP-2+DEX did not have a synergistic effect on fructose uptake, with values for the combination being equivalent to those achieved with GLP-2 alone. This suggests that the mechanism by which the treatments altered glucose and fructose uptake may have differed.

The changes in sugar uptake observed were not similar to the changes in lipid uptake observed in a parallel study (Iordache et al. 2004 unpublished data). For example, weanling animals whose dams were treated with DEX+GLP-2 showed consistent reductions in lipid uptake. This is in contrast to the increases in sugar uptake with GLP-2+DEX in weanling animals (Tables 30 and 31). Again, it appears that the treatments are having specific effects on the uptake of different nutrients.

Fructose and glucose uptake increased in the weanling animals more than one month after treatment of their pregnant and lactating dams with GLP2+ DEX (Tables 30 and 31). The mechanism of this late effect is unknown, but may have important nutritional implications. Metabolic programming is a phenomenon that refers to the late effect of early environmental influences (Barker, 1995).For example, epidemiological studies show that low birth weight is associated with increased risk of type 2 diabetes and the metabolic syndrome (reviewed in Ozanne and Hales, 2002). Recently, Jacobson and colleagues (2005) demonstrated that maternal dietary fat influences the composition of intestinal lipids and the responsiveness of the nursing offspring to experimental colitis.

Epigenetic modifications induced by early-life events may be responsible for the late effects observed in this study. For example, in a study exploring maternal-infant interactions, increased levels of pup grooming by rat dams within the first week of life was associated with altered DNA structure at a glucocorticoid receptor gene promoter in the hippocampus of the offspring (Meaney and Szyf, 2005). Differences in the DNA methylation pattern emerged and persisted into adulthood, with DNA methylation altering glucocorticoid receptor expression through modifications of chromatin structure. These findings demonstrate that the structural modifications of the DNA may occur as a result of early environmental influences. This mechanism has not been investigated in our model but remains an intriguing area of future research.

Data from animal studies also show that manipulating the maternal diet has lasting effects on the intestinal uptake of nutrients in the offspring (Karasov et al., 1985; Thomson and Keelan, 1987; Thomson et al., 1989; Keelan et al., 1990; Perin et al., 1997; Jarocka-Cyrta et al., 1998; Perin et al., 1999). Thomson et al. (1989b) altered the ratio of polyunsaturated to saturated fatty acids in the diet of weanling rats. They found that diets enriched in saturated fatty acids increased hexose uptake, and that these alterations were fast, progressive and irreversible. Feeding the same diets to pregnant and lactating rats resulted in similar increases in sugar uptake in their weanling offspring (Perin et al., 1997). Curiously, these changes were not seen in suckling offspring, suggesting that the mechanisms responsible for adaptation may not be fully developed in these animals. This phenomenon was also observed in our studies. Increases in uptake were seen in the weanling offspring of dams treated with DEX+GLP-2. However, these increases were not observed in the suckling (17-21 day old) offspring of the same dam (Drozdowski et al. 2005 unpublished work). This again suggests that the adaptive mechanisms are not fully functional in animals at this age.

The study by Perin et al. (1999) also noted that the pattern of adaptation appeared to differ between the jejunum and ileum. This was observed in our study as well. For example, while GLP-2+DEX increased jejunal fructose uptake, it had no effect on fructose uptake in the ileum (Table 30). Clearly there are site specific factors that influence the regulation of intestinal sugar uptake.

This study highlights the importance of the fetal and neonatal environment in the long-term health of an infant. This also has potentially important implications for the future health of infants whose pregnant or lactating mothers may have taken steroids or GLP-2 to treat medical conditions such as asthma, rheumatoid arthritis, inflammatory bowel disease or short bowel syndrome. Clearly, caution must be exercised in treating mothers with any agent that could alter the development of the offspring's intestine, and which could thereby potentially influence nutrient absorption and nutritional health in the future.

Increases in sugar uptake with GLP-2, DEX or GLP-2+DEX were observed in the jejunum and ileum of weanling animals (Tables 30 and 31), despite there being no significant changes in ileal morphology. This once again highlights the complex relationship between intestinal morphology and intestinal transport, and stresses that an alteration in intestinal morphology is not necessarily reflected by changes in absorption.

GLP-2 is trophic to the mature intestine (Drucker et al., 1996), and the GLP-2 receptor of mature animals is most abundant in the proximal small intestine (Munroe et al., 1999). The GLP-2 receptor is present in the fetal and neonatal gut, and responds to a degradation-resistant GLP-2 analog administered subcutaneously for ten days (Lovshin et al., 2000). Based on these observations, we expected to see increases in the intestinal villous height or crypt depth in GLP-2 treated animals. We did not see any significant effect of GLP-2 on intestinal morphology (Table 29), indicating that the route, dose and timing of our treatment did not produce a trophic effect on the weanling intestine.

Increases were seen in jejunal PCNA abundance with GLP-2, DEX and GLP-2+DEX (Figure 59A). However, glucose uptake was only increased with GLP-2+DEX, suggesting that there is not a direct relationship between proliferation and uptake. When jejunal apoptosis was assessed by a research pathologist (RREU), he found that all three treatments significantly reduced the apoptotic index (Table 32). The reduction in apoptosis was most pronounced with GLP-2+DEX, and this reduction coupled with the increase in proliferation may be partially responsible for the increase in glucose uptake. Still, fructose uptake was increased to a similar extent by both GLP-2 and GLP-2+DEX, suggesting that factors other than proliferation and apoptosis are influencing intestinal transport.

While DEX can pass through the placenta and is present in milk (Grosvenor et al., 1993), it is not known if the same is true for GLP-2. Several other growth factors and hormones are either produced by the placenta, or have receptors on the placenta such as IGF-1, IGF-II, and EGF (Fant, 1986; Scott et al., 1985; Shen et al., 1986; Maly and Luthi, 1986; Duello et al., 1994). Similarly, many hormones and growth factors are present in breast milk (Koldovsky and Goldman, 1999). Therefore, maternal GLP-2 may directly influence the offspring, or may influence other growth factors or hormones that can pass through or from the placenta, or into the breast milk, and thereby indirectly alter intestinal morphology or sugar uptake in the suckling animals. Furthermore, alterations in milk volume or changes in milk composition may have occurred in response to GLP-2 and DEX, although this was not investigated in this study.

Immunohistochemistry (IHC) was used to assess the protein abundance of sugar transporters SGLT1, GLUT2 and GLUT5. The abundance of the Na^+K^+-ATPase was also assessed, since it maintains the Na^+ gradient across the BBM which is necessary for the optimal functioning of SGLT1. A correlation between IHC staining and protein levels determined using other methods such as Western blotting (Venter et al., 1987; Podhajsky et al., 1997; Dias et al., 2000) or immunoassays (Aasmundstad et al., 1992; Lehr et al., 1997; Bhatnagar et al., 1999; Simone et al., 2000) has been shown. IHC has been used previously to demonstrate alterations in protein abundance in the intestine, such as with aging or a result of modifications in dietary lipids (Drozdowski et al., 2003a; Drozdowski et al., 2003b; Woudstra et al., 2004). GLUT2 is present in the BLM and functions to transport glucose and fructose out of the enterocytes (Thorens et al., 1988; Burant and Bell, 1992; Cheeseman, 1993). Under conditions of sugar loading GLUT2 may traffic to the BBM to augment sugar uptake (Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Gouyon et al., 2003). In these studies, IHC did not distinguish between the BBM or the BLM localization of GLUT2. In fact, GLUT2 in the jejunum was reduced with GLP-2+DEX, indicating that the IHC method used was sensitive enough to detect changes in protein abundance. We did not perform confocal microscopy in this study, so we do not know the exact location of the protein. In light of the reduction in sugar uptake, we speculate that the GLUT2 is largely intracellular and, therefore, non-functional. In the jejunum of weanlings given GLP-2, GLUT2 fell but fructose uptake increased (Table 30, Figure 58C). Thus, the transport alterations were not mirrored by changes in the abundance of GLUT2. Similary, changes in uptake were not explained by alterations in the abundance of SGLT1, GLUT5 or the Na^+K^+-ATPase.

The concept that there may be alterations in the intrinsic activity of a transporter is supported by the observation that there are discrepancies between glucose uptake and the protein abundance of glucose transporters in skeletal muscle (reviewed in Furtado et al., 2002), adipose tissue (Barros et al., 1997), and in the intestine (Maenz and Cheeseman, 1986; Corpe et al., 1996; Helliwell et al., 2000a; Au et al., 2002; Thiesen et al., 2003a; Drozdowski et al., 2003a; Drozdowski et al., 2003b). In the intestine, changes in the intrinsic activity of glucose transporters have observed with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000), and following the activation of MAPK and PI3K (Helliwell et al., 2000a).

Although we selected several signalling proteins that may play a role in the regulation of intestinal sugar uptake (Hirsch et al., 1996; Cui et al., 2003; Vayro et al., 1999; Veyhl et al., 2003; Helliwell et al., 2000a; Helliwell et al., 2003; Guan et al., 2003; Burrin et al., 2005), a number of these proteins including PKA, PKC, MAPK, p38 and GSK-3 failed to demonstrate significant changes with GLP-2 or DEX. However, both Akt and mTOR, members of the PI3K signaling pathway, were affected in this study. Helliwell et al. (2000a) showed that the PI3K pathway is involved in the modification of the intrinsic activity of GLUT2 and GLUT5. In this study, all three treatments significantly increased jejunal Akt abundance (Figure 3C). Based on Helliwell's results, one would expect an increase in Akt to produce an increase in GLUT2 and GLUT5 activity (fructose uptake), without increases in the abundance of these proteins. Indeed, when GLP-2+DEX were administered, the increase in jejunal Akt was associated with increased fructose uptake, while GLUT5 abundance was unchanged and GLUT2 abundance was actually decreased. Therefore, one may speculate that the changes in fructose uptake observed with GLP-2 and DEX are a result of PI3K-mediated changes in the intrinsic activity of GLUT2 and GLUT5. Similarly, the increase in the uptake of glucose seen with DEX+GLP2 in the jejunum of weanlings was associated with increased abundance of Akt (Table 31, Figure 3C). This supports the suggestion that these hormones alter the abundance and presumably the activity of Akt, which may in turn modify the intrinsic activity of BBM transporters. To investigate further the role of the PI3K pathway, we also determined the effect of DEX and GLP-2 on the abundance of mTOR, a downstream member of the PI3K pathway. In general, the changes seen in Akt (Figures 3C and 3D) were mirrored by parallel changes in mTOR (Figures 3E and 3F), further implicating the PI3K pathway.

Modifications in the intrinsic activity of SGLT1 via the PI3K pathway have not been documented. However, a study by Alexander and Carey (2001) showed that orogastric IGF-1 treatment increased glucose uptake in piglets without increasing SGLT1 abundance, suggesting an effect on intrinsic activity of the transporter. The increase in uptake was blocked by a PI3K inhibitor, implicating this pathway in the IGF-1 induced changes in uptake. We speculate that a similar mechanism may provide an explanation for the changes in glucose uptake observed in this study in response to DEX+GLP2.

Although we did not examine trafficking in our study, it is possible that the activation of the PI3K pathway is associated with an increase in the trafficking of the intestinal sugar transporters to the BBM. PI3K has been implicated in the regulation of GLUT4 trafficking to the plasma membrane in adipocytes or muscle (reviewed in Furtado et al., 2002). Still, several studies have shown that changes in intestinal sugar uptake seen after IGF-1, GLP-2 or glucose administration were not fully accounted for by alterations in the trafficking of transporter protein to the BBM (Alexander and Carey, 2001; Au et al., 2002; Khoursandi et al., 2004). Therefore, it is possible that both alterations in trafficking and intrinsic activity may have influenced sugar uptake in this study.

Increases in jejunal Akt and mTOR (Figures 3C and 3E) were associated with an increase in proliferation (Figure 3A) and a decrease in apoptosis (Table 32). Akt inhibits apoptosis by phosphorylating the pro-apoptotic protein Bad and by blocking cytochrome c release (Downward, 2004). mTOR may also regulate apoptosis as a member of an apoptotic pathway activated by microtubule damage and inducing phosphorylation of Bcl2 (Asnaghi et al., 2004). Furthermore, the PI3K/Akt pathway is thought to transduce proliferative signals from growth factor receptors to the cell cycle machinery of intestinal epithelial cells. Indeed, Akt increases cyclin D expression, promoting entry into the S phase (Sheng et al., 2003), while mTOR regulates several cell cycle proteins including pRb and $p27^{KIP1}$ (Asnaghi et al., 2004). Our data supports the view that increases in Akt and mTOR are associated with increases in proliferation and decreases in apoptosis in intestinal epithelial cells.

In conclusion, the maternal administration of GLP-2+DEX increases intestinal sugar uptake in weanling offspring. The mechanism of this late effect is unknown, but associated increases in Akt and mTOR suggest that alterations in the intrinsic activity of the sugar transporters may play a role. This study supports the concept that early exposure to maternal factors influences physiological functioning later in life and focuses attention on the importance of the fetal and neonatal environment.

ACKNOWLEDGEMENTS

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Table 29. The effect of treatment of pregnant and lactating rat dams with GLP2, DEX, or GLP2 + DEX on jejunal and ileal morphology of weanling rats

		CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	Villous height	149±49	212±4	219±15	203±9
	Villous width (base)	46±14	64±5	73±6	63±3
	Villous width (mid)	53±14	56±2	58±3	56±2
	Crypt depth	63±12	50±2	51±2	64±7
	Distance/5 villi	255±64	371±17	369±18	347±10
	Distance/5 cells	11±3	17±1	16±1	25±6
ILEUM	Villous height	288±44	310±77	263±19	239±17
	Villous width (base)	117±14	116±33	105±7	113±5
	Villous width (mid)	113±21	105±37	95±1	95±5
	Crypt depth	88±12	87±29	88±7	104±13
	Distance/5 villi	893±170	838±153	670±32	703±45
	Distance/5 cells)	42±7	31±4	26±1	27±2

Values are mean ± sem, n=8.
Distance measurements are represnted in microns.
The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation.

Table 30. The effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on fructose uptake in weanling rats

	CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	7.0±1.0 **a**	11.8±0.6 **b**	9.5±0.7 **ab**	11.6±1.3 **b**
ILEUM	12.5±0.8 **a**	11.7±0.8 **a**	8.5±0.6 **b**	11.8±0.8 **a**

Values are mean ± sem, n=8
Because fructose uptake is linear over the range of concentrations used, the data is presented as slopes of the lines
a, b: values with different letters are significantly different $p<0.05$ by ANOVA.
The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation. The weanlings were sacrificed on day 49.

Table 31. The effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on the Vmax and Km for glucose uptake in weanling rats

	Vmax	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	1624±316 **a**	1860±388 **a**	1472±129 **a**	3207±464 **b**
	Lineweaver-Burke	977±106 **a**	1079±93 **a**	1348±124 **a**	2033±120 **b**
	Wolfee	1590±166 **a**	1745±75 **a**	1490±106 **a**	2778±135 **b**
	Eadie-Hofstee	1115±121 **a**	1247±92 **a**	1393±117 **a**	2318±143 **b**
ILEUM	Sigmaplot	1050±184 **a**	1658±143 **a**	1283±102 **a**	3286±972 **b**
	Lineweaver-Burke	719±77 **a**	1330±180 **b**	979±11 **a**	845±71 **a**
	Wolfee	1112±101 **a**	1629±190 **b**	1227±57 **a**	2155±165 **c**
	Eadie-Hofstee	789±88 **a**	1471±181 **b**	1111±31 **ab**	1141±80 **ab**
	Km	**CONTROL**	**GLP-2**	**DEX**	**GLP-2+DEX**
JEJUNUM	Sigmaplot	22.2±10.2	27.4±12.6	21.4±4.5	50.9±13.2
	Lineweaver-Burke	5.9±0.0 **a**	7.7±0.4 **b**	18.5±0.4 **c**	23.1±1.6 **d**
	Wolfee	19.9±0.3 **a**	22.5±2.1 **a**	22.0±1.2 **a**	37.9±2.9 **b**
	Eadie-Hofstee	7.7±0.0 **a**	10.0±0.5 **b**	19.2±0.6 **c**	27.9±1.7 **d**
ILEUM	Sigmaplot	13.9±6.8 **a**	29.0±5.4 **a**	36.9±5.8 **a**	118.9±49.2 **b**
	Lineweaver-Burke	4.5±0.4 **a**	19.6±1.2 **b**	23.2±3.4 **b**	15.3±1.2 **b**
	Wolfee	15.8±0.1 **a**	27.4±0.7 **b**	33.1±2.5 **c**	62.2±3.7 **d**
	Eadie-Hofstee	5.4±0.6 **a**	22.7±0.9 **b**	27.9±3.2 **b**	22.1±2.2 **b**

Values are mean ± sem, n=8
Units: mM
a, b: values with different letters are significantly different $p<0.05$ by ANOVA.
The treatments include GLP-2 (0.1 μg/g twice a day), DEX (0.128 μg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation. The weanlings were sacrificed on day 49.

Table 32. The effect of treatment of pregnant and lactating rat dams with GLP-2, DEX or GLP-2 + DEX on the apoptotic index in the jejunum and ileum of weanling offspring

APOPTOTIC INDEX	CONTROL	GLP-2	DEX	GLP-2+DEX
JEJUNUM	2.2±0.2 **a**	0.7±0.1 **b**	0.3±0.1 **b**	0.2±0.1 **b**
ILEUM	2.3±0.3 **a**	0.9±0.2 **b**	0.5±0.1 **b**	1.1±0.2 **b**

Values are mean ± sem, n=2-3
*Apoptotic index=# of apoptotic cells/total number of cells*100*
a, b: values with different letters are significantly different $p<0.05$ by ANOVA.
The treatments include GLP-2 (0.1 µg/g twice a day), DEX (0.128 µg/g once a day), and GLP-2 + DEX at those doses, given during pregnancy and lactation. The weanlings were sacrificed on day 49.

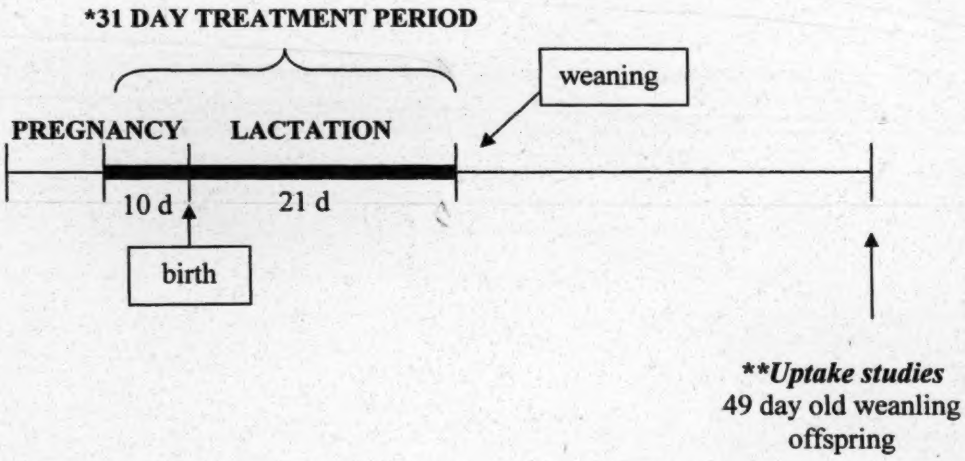
*31 DAY TREATMENT PERIOD
weaning
PREGNANCY
LACTATION
10 d
21 d
birth
**Uptake studies
49 day old weanling offspring

Figure 57. Experimental Design

*Treatment with GLP-2, DEX, GLP-2 + DEX, and Placebo was administered during the last 10 days of pregnancy and throughout lactation.

**Uptake studies were performed at day 49 ("weanling").

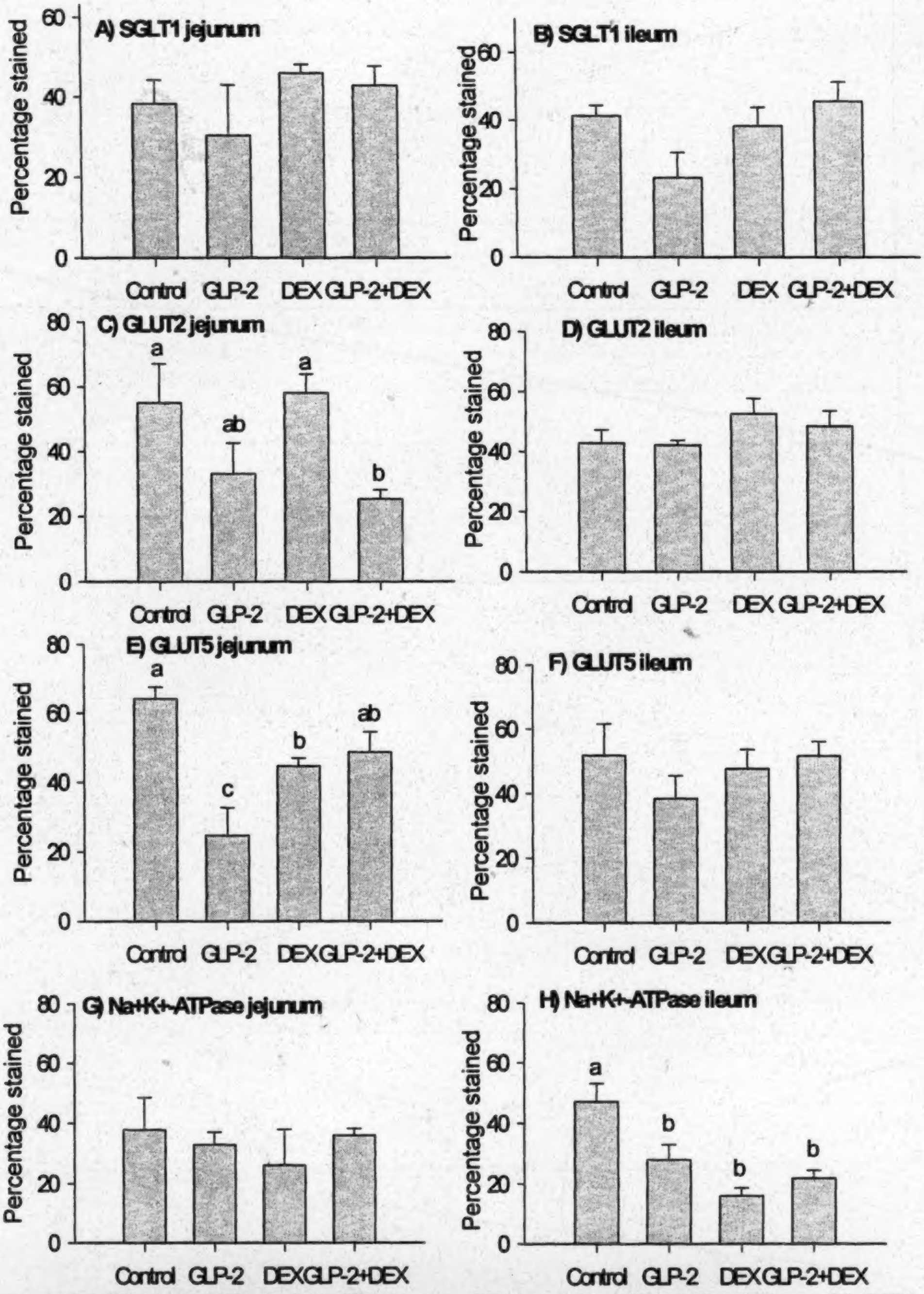

Figure 58. The Late Effect of the Treatment of Pregnant and Lactating Dams with DEX, GLP-2 and GLP-2+DEX on SGLT1, GLUT2, GLUT5, Na+K+-ATPase Protein Abundance As Determined by Immunohistochemistry.
Means+/-SEM, n=4-6, Bars with different letters are significantly different ($p<0.05$)

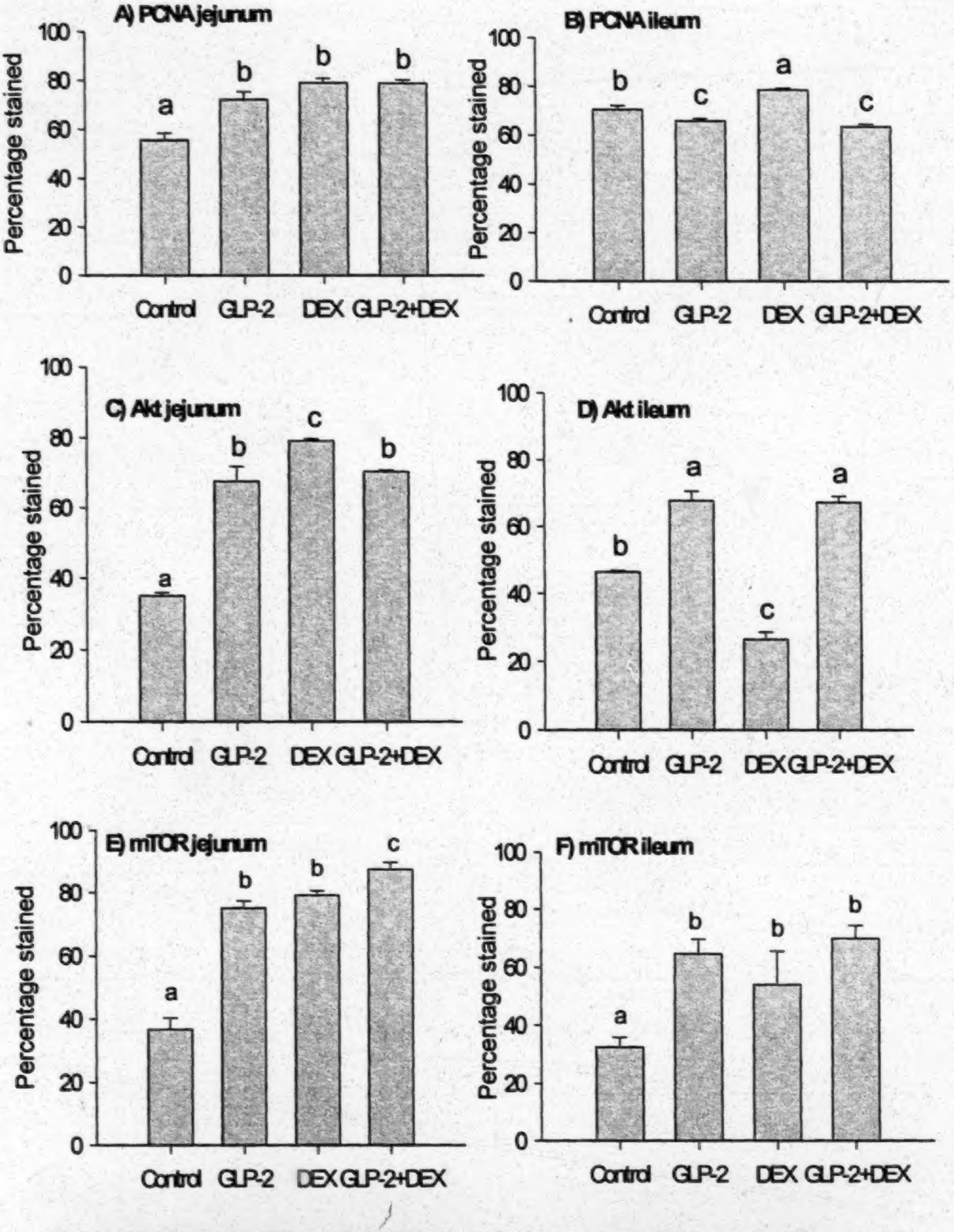

Figure 59. The Late Effect of the Treatment of Pregnant and Lactating Dams with DEX, GLP-2 and GLP-2+DEX on PCNA, Akt and mTOR Protein Abundance As Determined by Immunohistochemistry.

Means+/-SEM, n=3-4

Bars with different letters are significantly different ($p<0.05$)

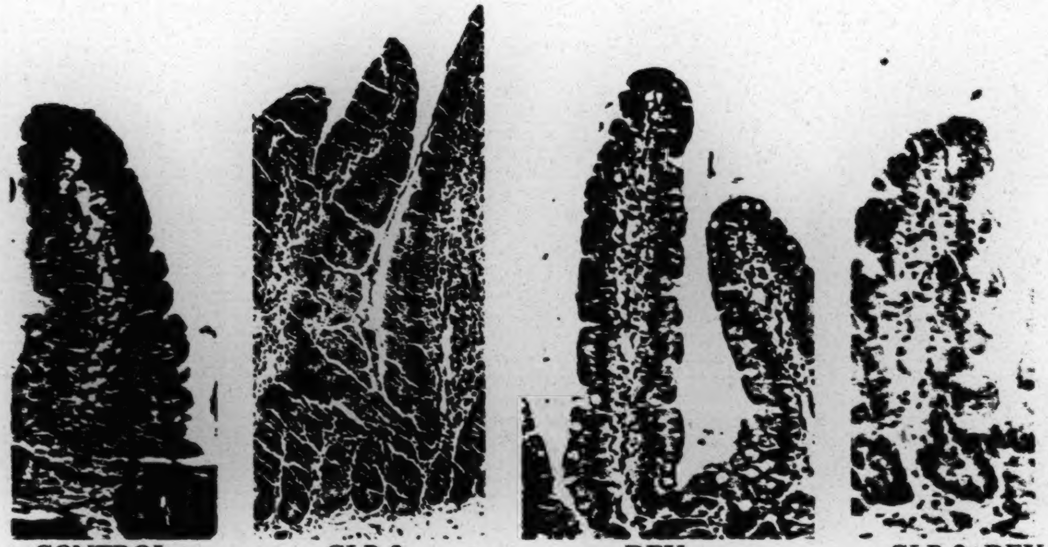
CONTROL
GLP-2
DEX
GLP-2+DEX

Figure 60. Representative immunohistochemistry images: GLUT2 abundance in the jejunum of weanling rats

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12. DISCUSSION

PHENOTYPIC CHANGES

12.1. AGING

Background

The purpose of my thesis was to examine the process of intestinal sugar uptake over the lifetime of the animal. As noted in my literature review (page 75), there are several ways to define aging. The term "ontogeny" is usually used to refer to early development, while the term "aging" is usually used to refer to the physiological processes that occur later in life. I have chosen to use the term "aging" to describe the age-related changes that occur throughout the lifespan of the animal from early development to senescence. The aging studies were done in two parts. The first, in which suckling (19-21 day old) and weanling (~49 day old) animals were studied, and the second in which young (1 month), mature (9 month) and old (24 month) rats were used. These descriptors (sucklings, weanlings, young, mature, and old) are used as a matter of convenience, without any implied judgment about the physiological significance of what is "young" "mature" or "old". It should be noted that in the second series of studies the young rats were 4 weeks of age, and were therefore younger than the 49 day old weanlings used in the first study. Thus, we cannot directly compare uptake results from the weanlings in the first and second studies. Instead we describe the qualitative changes in sugar tranport that occur from 3-100 weeks of age.

Because we were interested in the physiological and potential nutritional importance of the adaptive process that occurs with age, we began our studies by examining the phenotypic changes in intestinal sugar uptake. Once we observed significant alterations in uptake, we then sought to determine the mechanisms that might explain these changes, such as variations in intestinal morphology, transporter protein abundance, or intrinsic transporter activity. Thirdly, we sought to explain changes in uptake by exploring possible alterations in selected signals that might mediate the phenotypic alterations in transport. Our studies can be broadly divided into three groups: 1) the early and late development of intestinal sugar uptake; 2) the effect of GLP-2 and a selected glucocorticosteroid, dexamethasone (DEX) on intestinal sugar uptake early in life; and 3) the effect of isocaloric changes in the type of dietary lipids on intestinal sugar uptake later in life. The detailed descriptions of the individual studies are contained in the corresponding chapter (Chapters 4-11). This Summarizing Discussion will elucidate the way in which each manuscript is part of a puzzle that addresses the issue of intestinal sugar uptake, and its' regulation over the life of the animal.

When I started my thesis on the profile of intestinal sugar uptake over the lifespan of the animal, the published information in this area was limited. However, a collaborative study between our laboratory and researchers at McGill University (Wild et al., 2005, unpublished observations) profiled the mRNA expression and protein abundance during early development in neonatal rats (3-35 days of age). This study showed that glucose transporter (SGLT1, GLUT2) mRNA expression peaked at postnatal day 35, and that the protein abundance of the transporters followed a similar timeline. While this study did not measure sugar uptake, it was speculated that the transport protein was functional in these young animals. Others have suggested that glucose uptake in rats peaks shortly after birth (postnatal day 3), when the intestine takes over the burden of nutrient acquisition from the placenta (Toloza and Diamond, 1992). Kinetic studies done on pigs demonstrate that the maximal rates of transport for D-glucose were highest immediately after birth and even before suckling, with a subsequent decrease in absorption associated with the onset of suckling (Puchal and Buddington, 1992).

Although SGLT1 and GLUT2 are expressed both in the rat fetus and at birth, the expression of brush border membrane (BBM) GLUT5 is delayed, and is only significantly detected in post-weaning animals (Castello et al., 1995; Rand et al., 1993; Shu et al., 1997; Toloza and Diamond, 1992). In rats, GLUT5 protein and mRNA abundance parallel fructose transport, and therefore remain low throughout the suckling phase (day 1-14), with higher levels detected in the weaning phase (day 15-27) and post-weaning phase (day 28-42) (Toloza and Diamond, 1992; Rand et al., 1993; Castello et al., 1995). This increase in fructose uptake coincides with the appearance of fructose in the pups' diet, and parallels the increase in fructose uptake seen at this time. Although there is a temporal association between dietary fructose and the appearance of GLUT5 in the

BBM, the expression of the transporter is "hard wired" and occurs at this time even in the absence of dietary stimuli (Shu et al., 1998).

There are also changes in intestinal sugar transport in later life, but experiments using rodent models of aging demonstrate conflicting results. Several studies show reductions in D-glucose absorption in aged rats (Doubek and Armbrecht, 1987; Freeman and Quamme, 1986; Lindi et al., 1985). Depending upon the intestinal site studied, a normal or increased absorptive capacity was also found in a study using everted intestinal segments from old versus young rats (Darmenton et al., 1989). Results from studies in mice also do not offer conclusive results on the effect of aging on nutrient absorption. Ferraris et al. (1993) showed a reduction in uptake and site density of SGLT1 in aged mice. This is in contrast to the findings of Thompson et al. (1988), who showed an increase in intestinal glucose uptake in aged mice.

The uptake of fructose has also been studied in aging mice. Ferraris and Vinnekota (1993) showed that D-fructose uptake per milligram of tissue was higher in the jejunum of young as compared to old animals. Adaptive increases in uptake, in response to increases in dietary carbohydrate levels, were blunted in these mice, and were restricted to the more proximal regions of the small intestine.

Thus, while there are discrepancies between studies the literature generally supports the concept that there is an early decrease in intestinal sugar uptake, followed by a further decline later in life. The changes that occur early in life are not usually considered with the changes that occur in later life and *vice versa*. When I took this perspective and looked at the process that occurs over a lifetime, I developed the hypothesis that a continuum of a decline in intestinal sugar uptake occurs throughout the lifespan of the healthy rat.

Our data

Our data show a reduction in intestinal glucose and fructose uptake when comparing 49 day old post-weaning rats to 19-21 day old suckling rats (See Appendix: Table 35). As noted above, from a consideration of the literature this decrease in glucose uptake was expected. The decline in fructose uptake, which we observed between sucklings and weanlings, was initially puzzling to us, as based on the work of Toloza and

Diamond (1992) one might have expected higher levels post-weaning. However, these researchers measured fructose uptake in suckling rats at 1-14 days of age and in weanling rats at 15-27 days of age. Therefore, it is likely that our 19-21 day old "suckling" rats were actually equivalent in age to the animals described as weanlings in this previous study. Therefore, it is likely that we missed the initially lower rates of fructose uptake described by these researchers in 1-14 day old rats. Indeed, we measured fructose uptake in 19-21 day old rats, a time at which the animals are in the process of weaning. The high levels seen at this time may indicate an anticipatory hard-wired increase in fructose uptake, as described by Shu et al. (1998).

We also chose to look at the effect of age on intestinal sugar transport later in life, in order to determine if there are potentially significant reductions in uptake associated with senescence. Our data showed that there were significant declines in both glucose and fructose uptake when comparing 1 and 9 month old animals (Figures 12,13,23,24). Interestingly, no further decreases were seen in 24 month old animals, indicating that in this study senescence is not associated with a further decline in intestinal sugar transport. Thus, the decline in sugar uptake that begins at suckling continues to midlife after which uptake rates remains relatively stable.

12.2. GLP-2/DEXAMETHASONE

Background

Having established a continuum of decline for sugar transport over the lifetime of the animal, we then sought to determine if this process could be modified. There is an extensive literature on the process of intestinal adaptation (reviewed in Thiesen et al., 2003a). Sugar uptake is up-regulated in diabetes (Keelan et al., 1985), following intestinal resection (Robinson et al., 1982), or by feeding a diet enriched in saturated fatty acids (Thomson et al., 1986;1987;1988). Conversely, intestinal sugar uptake is down-regulated with the use of total parenteral nutrition (Inoue et al., 1993), abdominal radiation (Thomson et al., 1983), and by feeding a diet enriched in polyunsaturated fatty acids (Thomson et al., 1986;1987;1988). There are two main approaches to physiologically alter intestinal nutrient absorption: 1) vary the amount or type of nutrients in the diet; or 2) administer selected hormones or growth factors known to influence this process. Because our laboratory has already published studies looking at the effect of early dietary changes on intestinal transport (Perin et al., 1997; Jarocka-Cyrta et al., 1998; Perin et al., 1999), we chose to take a novel and potentially clinically significant approach, and look at the influence of a glucocorticosteroid and glucagon-like peptide 2 (GLP-2) on the ontogeny of intestinal sugar transport. There is considerable literature on the effect of steroids on the precocious induction of BBM enzymes (Batt and Scott, 1982; Martin and Henning, 1984). Steroids have been previously shown to increase sugar uptake in adult animals (Thiesen et al., 2002; Thiesen et al., 2003b; Thiesen et al., 2003c). However the influence of steroids on intestinal transport when given directly to young suckling animals is unknown. Also, it is not known if maternal steroids influence intestinal transport in their offspring, or if any potential effects persist into later life.

Although we have chosen to use DEX in this study, we acknowledge that it is not commonly used in humans due to its adverse effects (Joint Statement, Canadian Pediatric Society, 2002). Still, Dex is commonly used in the rodent research setting. Unlike most steroids, which are bound in plasma to the corticosteroid binding globulins (CBG), Dex circulates freely, with blood concentrations unaffected by changes in CBG levels. This is particularily important in studies of development, as CBGs are developmentally regulated (D'Agostino and Henning, 1981). Furthermore, the unique structure of Dex allows it to cross the placenta by escaping deactivation by 11β-HSD_2. It is for these reasons that we have chosen to use Dex in our studies of early development. We acknowledge that these results cannot be directly related to the human neonatal situation, since DEX would not be given in a clinical setting because of the adverse effects associated with this steroid.

GLP-2 increases intestinal morphology and sugar transport in adult animals (Drucker et al., 1996; Tsai et al., 1997; Cheeseman and Tsang, 1996; Cheeseman, 1997; Cheeseman and O'Neill, 1998). Thus, both GLP-2 and DEX stimulate intestinal sugar uptake. The role of GLP-2 in the ontogeny of sugar transport, however, has not been established. Accordingly, we hypothesized that the normal developmental decline of intestinal sugar transport may be prevented by GLP-2 and dexamethasone (DEX).

Our data

We chose three approaches to examine the effect of GLP-2 and DEX on intestinal sugar uptake in young animals. The first was to administer these agents for ten days to suckling rats, and to examine uptake immediately after completion of the ten day course, as well as four weeks later. This allowed us to examine the direct effect of GLP-2 and DEX on sugar uptake in sucklings, and the possibility of a persistent effect, or perhaps even the development of a new response. We recognized the possibility that ten days of treatment might be too short, or that suckling rats may have been too young to respond with the expected stimulatory effect.

Sucklings treated directly with GLP-2 or DEX did not demonstrate changes in sugar uptake (Table 18,19,20), possibly due to the timing or duration of treatment. Wild et al. (2005, unpublished observations) showed that administering spermidine to 3-7 day old rats did increase sugar transporter protein. However, uptake was not measured in their study, so it is not certain if the increased protein that they measured was functional. We believe that ten days of treatment should have been sufficient to elicit a response in sucklings, since morphological responses were seen, despite there being no changes in transporter abundance or sugar uptake. It still remains a possibility, however, that while the morphology was capable of responding, the sugar transporters were not capable of activation in response to these stimuli in these young animals. There is evidence that the intestine of suckling animals is unable to modify intestinal nutrient transport in response to certain dietary manipulations (Toloza and Diamond, 1992; David et al., 1995; Shu et al., 1997). In fact, some investigators have suggested that the ability of the intestine to modify transport is acquired at weaning, when the composition of the diet becomes more variable (Jiang et al., 2001). This is an interesting theory that requires further clarification, but our own published data showing the adaptation of nutrient transport when the type of lipids is varied in the diet of the mother (Jarocka-Cyrta et al., 1998) argues that the intestine of suckling rats is capable of adapting in response to some stimuli. Still in this current study, the suckling intestine was capable of responding, for example by altering its' morphology in response to DEX and GLP-2.

One possibility is that these stimuli may have to be applied earlier in life, at a critical period, or for a longer duration, in order to alter ontogeny. This concept is the basis for our decision to perform further studies investigating the effect of maternal DEX and GLP-2 on the offspring. Also, as previously mentioned, our laboratory has shown that maternal diet influences transport in the offspring (Perin et al., 1997; Jarocka-Cyrta et al., 1998). Thus, we know that the suckling intestine is capable of adaptation during this period, at least in response to dietary lipids.

In contrast to the first "Young Study" discussed above, in the second "Lactation Study" (Chapter 9) GLP-2 and DEX were given to lactating rat dams (for 21 days throughout lactation), and intestinal sugar uptake was assessed in their suckling and weanling offspring. In this case, GLP-2 and DEX produced few significant changes in sugar uptake. We speculate that this relative lack of effect was due either to a lack of responsiveness of the intestine to GLP-2 or DEX when co-administered at this time, or again was due to an insufficient duration of co-administration of GLP-2 and DEX. Accordingly, in the third "Pregnancy Study" (Chapters 10 and 11), we maximized the length of treatments to determine if the intestine was capable of responding to these agents. When GLP-2 and DEX were given to dams during pregnancy and lactation (for the last 10 days of pregnancy and throughout the 21 days of lactation), these agents surprisingly decreased intestinal sugar uptake in the suckling offspring. This was initially puzzling, but of course one must remember that there is a normal ontogenic decline in sugar uptake. Therefore, the inhibitory effect of GLP-2 and DEX on sugar uptake may represent a precocious induction of the normal ontogenic decline.

The results of these three studies suggest that the duration of the stimulation with GLP-2 and DEX must be sufficiently long, and perhaps must include a critical period during pregnancy. It also appears that there is a late effect to the early administration of these agents. This phenomenon will be discussed in the next section.

Late Effects

There are many studies that demonstrate critical period programming in the intestine (Perin et al., 1997; Perin et al., 1999, Jarocka-Cyrta et al., 1998; Jacobson et al., 2005). Because we saw decreases in sugar uptake in the suckling offspring of dams treated with DEX and GLP-2 during pregnancy and lactation ("Pregnancy Study"), we wanted to determine if these effects persisted in later life. Because GLP-2 and DEX enhance uptake in mature animals, we had hypothesized that these agents would prevent the age-associated decline in intestinal sugar uptake. This in fact did occur. For example, GLP-2 and DEX increased glucose uptake in the weanling offspring, a month after their administration had ceased. This abolished the usual decline in glucose uptake that occurs between the suckling and weanling period. The results indicate that the effect of these agents varies with age, and supports the suggestion that the intestine of young animals is capable of modifying sugar transport in response to GLP-2 and DEX.

In this study GLP-2 and DEX decreased glucose uptake in sucklings with high uptake rates, and increased glucose uptake in weanlings who had lower uptake rates. Is it possible that GLP-2 and DEX can modulate uptake in either direction depending on the initial rate of uptake? This is an interesting possibility, because usually uptake falls between suckling and weanling. However, in this study DEX and GLP-2 increased uptake in weanlings. Therefore, this does not appear to be a precocious induction of the normal ontogenic decrease, but perhaps is indicative of a reprogramming of the normal ontogenic process. Epigenetic modifications induced by early-life events may be responsible for the late effects observed in this study. For example, early life events have been associated with altered DNA structure and differences in DNA methylation patterns (Meaney and SZyf, 2005). This mechanism has not been investigated in our model but remains an intriguing area of future research.

This effect of GLP-2 and DEX was specific to sugar uptake, as lipid uptake in sucklings was decreased by maternal DEX and GLP-2 (Iordache et al., 2005, unpublished observations), and this effect persisted into the post-weaning period. In this latter instance, one may speculate that GLP-2 and DEX produced a precocious induction of the normal age-related decline in lipid uptake. It is not known why these differences exist between the effect of GLP-2 and DEX on lipid and sugar uptake. Clearly this is not an effect that can be generalized to all nutrients.

The normal decline in sugar uptake between suckling and weanling was prevented by DEX and GLP-2, and uptake rates in weanlings were restored almost to suckling values. This may have important clinical implications. Even if this late effect is lost in later life, these changes during this critical period may potentially have serious long-term nutritional consequences.

It is not known if GLP-2 crosses the placenta or if it passes into the mother's milk. Therefore, it is not clear if the intestinal effects seen with GLP-2 are direct or indirect. In contrast, DEX does cross the placenta and passes into milk (Grosvenor et al., 1993; Reece et al., 1995), and thus the effects of the administration of this steroid may be due to the steroid itself. Further research is needed to determine a) if GLP-2 is present in breast milk; b) if it crosses the placenta, and c) whether the effect of GLP-2 on the intestine when administered during pregnancy and lactation is a direct effect of the peptide, or an indirect effect due to the stimulation of other factors.

Indeed, there is evidence of interactions between GLP-2 and other hormones and growth factors. Drucker et al. (1997) examined the effect of a combination of factors (including GLP-2, EGF, IGF-I, IGF-II, and GH) on intestinal morphology. Mice treated with GLP-2 and either GH or IGF-I exhibited increased small bowel growth than did mice treated with GLP-2 alone. The investigators speculated that endocrine factors and epithelial growth factors may be intestinotrophic due to the stimulation of other trophic factors. While the Drucker study failed to provide direct evidence of this occurrence, it did show that mice treated with epidermal growth factor (EGF) exhibited a decrease in intestinal proglucagon-derived peptides, suggesting that there are interactions between these factors.

Kitchen et al. (2005) showed that GLP-2 and EGF produced synergistic effects on intestinal growth in parenterally fed rats. The mechanism responsible for this effect was not determined. However, previous reports have shown that GLP-1, a peptide closely related to GLP2, transactivates the EGF receptor (Buteau et al., 2003). Furthermore, data from a study by Orskov et al. (2005) suggests that GLP-2 acts by activating receptors on the subepithelial myofibroblasts, causing the release of growth factors such as KGF, which in turn stimulate intestinal growth. The effect of GLP-2 on other hormones and growth factors and the cross-talk between signals downstream of the receptor should be further characterized in future studies.

In this study we chose to examine the effects of combining GLP-2 and dexamethasone. We hypothesized that GLP-2 and DEX may interact to produce a synergistic effect on nutrition absorption in suckling animals, as glucocorticoids have a permissive effect on hormones, such as GLP-2, which act on G-protein coupled receptors and increase adenylate cyclase (Michel et al., 1994; Meier, 1997). There are many examples from our data showing that a combination of these factors has a more potent effect than either agent alone. For example, in the "Pregnancy Study", when compared to controls (3710±322) glucose uptake was reduced to a greater extent by GLP-2+DEX (1892±171) than by GLP-2 (2910±340) or DEX (2290±335) alone. Conversely, when compared to controls (1624±316) jejunal glucose uptake in weanlings was increased to a greater extent by GLP-2+DEX (3207±464) when compared to GLP-2 (1860±388) or DEX (1472±129) alone. Therefore, our data suggests that combining these two factors produces a more pronounced effect on intestinal sugar transport than does either agent alone.

It is important to acknowledge that we did not consider the impact of potential litter effects on our data. Because each of the offspring of any given dam was exposed to the same treatment regime, it is possible that differences observed between groups is partially due to differences between the litters. Of course, this is an important issue that could only be overcome by either cross-fostering the offspring or by including litter as a random nested factor in the statistical analysis. This issue is discussed in detail by Wainwright (1998). She points out the limitations of using this type of experimental design, and suggests alternative ways in which to obtain data from litters of animals. Clearly future studies would need to address these concerns in order to optimize the validity of the results.

12.3. DIETARY LIPIDS

Background

After establishing the age-associated decline in intestinal sugar uptake, as well as characterizing the effect of GLP-2 and DEX in early life, we then sought to determine the effect of dietary lipid modification on intestinal sugar uptake later in life. There is extensive literature on the adaptation of the intestine in response to alterations in both the amount and the type of nutrients in the diet. For example, there are increases in intestinal nutrient uptake with high fat diets (Sukhotnik et al., 2003), high CHO diets (Ferraris et al., 1993; Ferraris and Diamond, 1997) and early fructose feeding (Shu et al., 1998). In adult animals, SFA increases sugar uptake when compared to PUFA (Thomson et al., 1986; 1987; 1988), and feeding saturated fatty acids to pregnant dams increased sugar uptake in their weanling offspring (Perin et al., 1997). Until I began my studies, however, it was not known if we could apply this information obtained in younger animals to older animals.

The effect of aging on intestinal adaptation is unclear. Some investigators have suggested that the ability to adapt is reduced in aging. For example, Ferraris and Vinnekota (1995) showed that the intestinal adaptive increases in response to a high carbohydrate diet were reduced in old animals, and changes were limited to the proximal small intestine. Other studies have demonstrated that responsiveness to dietary changes is not lost with aging. Following 3 days of starvation, aged animals demonstrated an exaggerated enzyme response (lactase, sucrase, maltase) to refeeding (Holt and Kotler, 1987). Because when I began my studies there was no clear evidence that age impairs the response of the intestine to dietary lipids, we hypothesized that, as is the case in adult animals, the intestinal uptake of sugars in aged animals would be increased by feeding a SFA diet.

Our data

In this study, intestinal sugar uptake (based on intestinal weight, mucosal weight or serosal weight) at 1, 9 or 24 months was not significantly different between animals fed a PUFA or SFA diet (Figure 33,43). However, when uptake was expressed on the basis of mucosal surface area, sugar uptake was actually increased by PUFA when compared to SFA. This is opposite to what is seen in younger animals. A discussion of the paradoxical changes in intestinal sugar uptake in response to feeding PUFA follows in Section 12.4.

Having characterized the phenotypic changes in intestinal sugar uptake with age, GLP-2 and DEX, and with dietary lipids, we next endeavored to elucidate the mechanisms responsible for these alterations.

12.4. MORPHOLOGY AND TRANSPORTER ABUNDANCE

Our fourth hypothesis was that the changes in sugar uptake associated with age, GLP-2 and DEX, and dietary lipids are associated with parallel changes in intestinal morphology and transporter abundance. I will now discuss whether or not this hypothesis was confirmed in each of these models.

MORPHOLOGY

Aging

It is common to express rates of uptake on the basis of the weight of the intestine. If mucosal mass is increased, if there is greater surface area, or more transporter on the villus, then the age-associated decrease may simply be due to these morphological alterations, rather than on the basis of changes in the intrinsic properties of the transporters.

We explored the possibility that the declines seen in glucose and fructose uptake with aging may associated with concomitant decreases in intestinal morphology. Our data does not support this morphological explanation. For example, there were significant increases in intestinal morphology between suckling and weanling rats, a time when uptake fell (Chapter 10 and 11). Furthermore, there were no changes in morphology between 1, 9 and 24 month old rats, despite there being significant decreases in uptake between 1 and 9 month old rats (Figures 12,13,23,24).

GLP-2/Dexamethasone

As with the previously mentioned aging studies, the changes in sugar uptake with GLP-2 and DEX were not explained by alterations in intestinal morphology. For example, in the Pregnancy Study, jejunal sugar uptake in sucklings was reduced (Tables 27 and 28) with GLP+DEX, while intestinal morphology was unchanged (Table 26). In addition, GLP-2+DEX increased sugar uptake in weanlings (Tables 30 and 31), without significantly affecting intestinal morphology (Table 29).

Dietary Lipids

The influence of dietary lipids on intestinal morphology at 1, 9 and 24 months was examined. Significant changes in mucosal surface area were seen in SFA fed rats, with a large decrease at 9 months when compared to 1 month (Figure 31). Curiously, the 24 month old animals did not demonstrate a further decline, and actually had increased mucosal surface area when compared to their 9 month old counterparts. In PUFA fed animals, intestinal morphology was similar at 1 and 9 months, and then decreased significantly in the 24 month old animals. We interpret this to mean that feeding PUFA delayed the loss of mucosal surface area that occurred with the SFA diet. In the 9 month old PUFA fed animals, mucosal surface area was increased when compared to their SFA fed counterparts. This agreed with previous work that showed that polyunsaturated fatty acids increased ileal mucosal surface area in adult rats (Thomson et al., 1986). In contrast, the 24 month old PUFA fed rats had reduced mucosal surface area when compared to their SFA fed counterparts. This demonstrates that age influences the trophic response that certain dietary lipids have on the young intestine. This also reemphasizes the point that the results of an adaptive challenge described in younger rats do not necessarily apply to older animals. The pleiotropic or diverse effect of dietary lipids at different ages is a novel and intriguing finding.

These large changes in intestinal morphology had important effects on sugar uptake. When uptake was expressed on the basis of intestinal weight, mucosal weight or serosal weight, there were no significant changes between SFA and PUFA fed rats. However, when one expressed uptake per unit of mucosal surface area, there were now significant differences in sugar transport with diet (Figures 34 and 45). The results show that at 1 month of age there is no difference in glucose or fructose uptake between SFA and PUFA fed animals. Previous studies have shown that feeding SFA, as compared to PUFA, to pregnant rat dams during pregnancy and then weaning the offspring onto the same diet for 2 weeks, results in increases in glucose uptake. In our current study the animals were weaned onto SFA for 2 weeks, resulting in no significant changes in sugar uptake. Thus, it appears that the maternal feeding of SFA may have been responsible for the previously observed changes in sugar uptake. Again, this highlights the importance of the fetal environment on subsequent intestinal function.

In 9 month old animals, sugar uptake was increased by a SFA diet when compared to PUFA. This is in agreement with previous studies done in our laboratory demonstrating that feeding an SFA diet to adult rats increases sugar uptake (Thomson 1986;1987;1988). In direct contrast, in 24 month old animals sugar uptake was increased by PUFA when as compared to an SFA diet. This demonstrates that age has a significant effect on the intestinal response to dietary lipids.

The changes in uptake were related to alterations in intestinal morphology. Due to the smaller mucosal surface area in 9 month SFA fed rats, the uptake per unit mucosal surface area was actually very high. Similarly, the low amount of mucosal surface area in 24 month old PUFA fed animals resulted in higher levels of uptake per unit mucosal surface area. In both of these cases, it appears that this reduced mucosal surface area was very active, and was transporting sugars at a very high rate, possibly to compensate for its reduced area.

We understand that there is a potential limitation of our arguments suggesting that there is an obvious link between sugar uptake and mucosal surface area. We must acknowledge that we did not measure the microvillous surface area in these studies. There is evidence that adaptations in intestinal sugar uptake may also be associated with alterations in microvillous morphology. For example, EGF-induced increases in sugar uptake is associated with increased height of the microvilli (Hardin et al., 1999), presumably resulting in a greater mucosal surface area. We have performed a pilot study to determine the effect of age and dietary lipids on microvillous height. Although we are unable to perform a statistical analysis on these limited results (n=1), the values suggest that there may be reductions in microvillous height at later ages. Future work should include these important measures as additional indexes of intestinal morphology.

In summary, the age-and hormone-associated changes in intestinal sugar uptake were not associated with parallel alterations in morphology. In contrast, dietary lipid composition did significantly affect intestinal morphology. The changes in morphology did not parallel changes in sugar uptake, but instead illustrated that alterations in the activity of the transporters was occurring, possibly to compensate for the changes in mucosal surface area.

TRANSPORTER ABUNDANCE

Aging

The rate of glucose and fructose uptake could decrease with age if there was a fall in the abundance of transporter protein. In order to determine if decreases in uptake were paralleled by reductions in the abundance of sugar transporters, we used immunohistochemistry (IHC) to investigate the effect of age on the abundance of SGLT1, GLUT2, GLUT5 and $Na^{+}K^{+}$-ATPase. A correlation between IHC staining and protein levels determined using other methods such as Western blotting (Venter et al., 1987; Podhajsky et al., 1997; Dias et al., 2000) or immunoassays (Aasmundstad et al., 1992; Lehr et al., 1997; Bhatnagar et al., 1999; Simone et al., 2000) has been shown. IHC has been used previously in our laboratory to demonstrate alterations in protein abundance in the intestine, such as with aging, steroids, or a result of modifications in dietary lipids (Thiesen et al. 2003b; Woudstra et al., 2004). Using this method, we did not find significant age-related changes in SGLT1, GLUT5 or GLUT2 abundance. Because $Na^{+}K^{+}$-ATPase maintains the sodium gradient necessary for the functioning of SGLT1, we looked for changes in the abundance of this protein as a way of possibly explaining the changes in glucose uptake. No significant age-related changes were seen. For example, there was a reduction in intestinal glucose uptake (expressed on the basis of intestinal weight) between 1 and 9 month old rats. However, there were no concomitant changes in the abundance of SGLT1, GLUT2 or $Na^{+}K^{+}$-ATPase between those two age groups. This indicates that the abundance of these proteins was not responsible for the changes in uptake that we observed.

GLP-2 and Dexamethasone

As with the previously mentioned studies, the changes in sugar uptake with GLP-2 and DEX were not explained by alterations in transporter protein. For example, in the Pregnancy study, despite increases in intestinal sugar uptake with GLP-2+DEX (Tables 30 and 31) in weanlings, no changes in the abundance of SGLT1, GLUT2 or GLUT5 were detected using immunohistochemistry (Figure 58).

Dietary Lipids

Similarly, the changes in sugar uptake with alterations in dietary lipids were not explained by alterations in transporter protein. For example, despite increases in ileal glucose uptake in 24 month old animals fed PUFA (as compared to SFA) (Figure 34), transporter abundance was unaffected by this diet (Figures 35-38).

In summary, this adaptation in the rates of sugar uptake was not associated with significant changes in the abundance of sugar transporters. However, if these values were expressed per unit of mucosal surface area, we would clearly see that the activity of these proteins were increased when mucosal surface area was low (possibly to compensate) and were decreased when mucosal surface area was high (possibly to reduce biosynthetic costs and to closely match uptake capacity to dietary intake). This is in keeping with Diamond (1991) who uses the phrase "enough, but not too much" to describe the matching of intestinal sugar transport to dietary load.

Thus, while protein abundance did not change in this study, the activity of the transporters may still have been modified. This concept will be discussed further in the section on '***INTRINSIC ACTIVITY***' (page 420).

12.5. ALTERNATIVE MECHANISMS

We have not confirmed the hypothesis that age-, hormone- and diet-associated alterations in sugar uptake are due to reductions in the protein abundance as we have shown a disassociation between protein abundance and function. Clearly, some other mechanism is responsible for these age-related reductions in sugar uptake. A number of alterative mechanisms exist, that could possibly explain the age- and hormone-related changes in intestinal sugar uptake (Figure 62). These might include variations in the distribution of GLUT2, as well as other transporters within the enterocyte, the distribution of the transporters along the villus, or the intrinsic activity of the transporters.

GLUT2 in BBM

GLUT2 is present in the basolateral membrane (BLM), and functions to transport glucose and fructose out of the enterocytes (Thorens et al., 1988; Burant and Bell., 1992; Cheeseman, 1993). Under conditions of sugar loading, GLUT2 may traffic to the BBM to augment sugar uptake by SGLT1 and GLUT5 (Kellett and Helliwell, 2000; Helliwell et al., 2000a; Helliwell et al., 2000b; Gouyon et al., 2003). In our studies, IHC did not distinguish between the BBM or the BLM localization of GLUT2. Nonetheless, our results show that there were no changes in total GLUT2 protein. Furthermore, in the aging studies we performed Western blots on BLM proteins, and also saw no changes in GLUT2 abundance. Using a standard protocol for membrane isolation and blotting, we were unable to detect GLUT2 in the BBM under our experimental conditions, where the animals were fed, but were not challenged with a high sugar load. Future studies should investigate the effect of age, hormones and diet on the recruitment of GLUT2 to the BBM after a sugar challenge. The relationship between GLP-2 and the recruitment of GLUT2 to the BBM is of particular interest, considering the finding that vascular infusions of GLP-2 promote the rapid insertion of GLUT2 into rat jejunal BBM (Au et al., 2002).

INTRACELLULAR AND MEMBRANE DISTRIBUTION

Another possibility is that alterations in the distribution of transporter protein between the cytosol and the membrane may have occurred. Furthermore, even in studies where BBM was isolated, it is possible that the transporter was in a location within that membrane that discouraged access of the sugar in the lumen to the BBM transporter. We have not performed confocal microscopy in this study, so we are not able to comment on whether the alterations in sugar uptake observed in our studies are due to the movement of transporter from a position in the membrane where transport is maximal.

It is not known if there are intracellular pools of pre-formed pools transporters. Takata et al. (1992) failed to identify an internal pool of SGLT1 using immunogold staining. However, the work of Chung et al. (2002) suggests that EGF upregulates intestinal glucose transport by stimulating the translocation of SGLT1 from a microsomal pool to the BBM. Treatment with EGF increased SGLT1 protein in BBM vesicles and reduced the SGLT1 content in microsomal membrane fractions (Chung et al., 2002). In addition, EGF-stimulated glucose transport requires actin polymerization (Chung et al., 1999) and is associated with increases in brush border membrane surface area (Hardin et al., 1993). There is also unconfirmed, unpublished preliminary evidence from human ileal biopsies observed at colonoscopy that there may be an intracellular pool of SGLT1

(Wild et al., unpublished observations, 2005). In addition, the trafficking of GLUT2 to the BBM has been described under three conditions: sugar loading by gavage or diet (Helliwell and Kellett, 2000b; Gouyon et al., 2003); in experimental diabetes (Corpe et al. 1996); or as a result of vascular infusions of GLP-2 (Au et al., 2002). Therefore, the identification of intracellular pools of transporters, and the role of trafficking in the regulation of intestinal sugar uptake has yet to be established.

DISTRIBUTION ALONG CRYPT-VILLOUS AXIS

Transporter protein may be expressed equally along the villus, but is usually active in the upper third region of the villi (Ferraris et al., 1992). In diabetes, for example, there is a "recruitment" of transporters lower on the villus (Fedorak et al., 1991) that may partially explain the increases in glucose uptake seen in diabetic animals. We looked at the distribution of SGLT1 and GLUT5 along the CV axis, and did not see changes in protein distribution with aging (data not shown). The protein was evenly distributed along the villous and this was not influenced by age. This was also the case in the studies using GLP-2, DEX and dietary lipids. While we have looked at the protein distribution in this study, phloridzin-binding experiments could be done in the future to determine the activity of the proteins along the crypt-villous axis. However, based on our data we do not suggest that the decline in sugar uptake with age, or the changes in sugar uptake with GLP-2, DEX or dietary lipids was due to a change in the distribution of transporter along the villus.

We acknowledge that in many studies reported in the literature, transporter abundance along the villus does not necessarily reflect activity (Ferraris et al., 1992). We propose that the intrinsic activity of the individual transporters was modified by aging and thereby was responsible for decreases in sugar uptake. This concept will be discussed below.

INTRINSIC ACTIVITY

Although we have some evidence that the effect of dietary lipids on intestinal sugar uptake is associated with changes in intestinal morphology, the mechanism responsible for the age-, hormone- and diet-mediated changes in intestinal sugar transport remains to be elucidated. As noted above we have witnessed an apparent dissociation between transporter protein abundance and function throughout our studies. Indeed, there has been a long history of reports of discrepancies between glucose uptake and the protein abundance of glucose transporters in skeletal muscle (reviewed in Furtado et al., 2002), adipose tissue (Barros et al., 1997) and in the intestine (Maenz and Cheeseman, 1986; Corpe et al., 1996; Helliwell et al., 2000a; Au et al., 2002; Thiesen et al., 2003b; Drozdowski et al., 2003a; Drozdowski et al., 2003b). Changes in the intrinsic activity of sugar transporters have been observed with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000), and following the activation of MAPK and PI3K (Helliwell et al., 2000a).

The post-translational mechanism by which the intrinsic activity of intestinal sugar transporters is regulated is not known, but may involve phosphorylation of the transporter (Hirsch et al., 1996; Ishikawa et al., 1997), the activation or inhibition of the transporter by a regulatory protein (Vehyl et al., 1993; Vehyl et al., 2003), or as noted above, the position of the transporter in the membrane and the its microenvironment. We propose that a fall in the intrinsic activity of the transporter may represent the mechanism responsible for the age-associated decline in sugar uptake, as well as the changes in intestinal sugar uptake observed with GLP-2 and DEX administration and alterations in dietary lipid composition. The intracellular signals involved in regulating the intrinsic activity of transporters have not fully been characterized. However, we have chosen to investigate several signals known to regulate sugar transport in various models.

12.6. SIGNALLING PROTEINS

We hypothesized that the decline in intestinal sugar uptake over the lifespan, and the changes in sugar uptake with GLP-2, DEX and dietary lipids will be associated with parallel changes in the abundance of selected signaling proteins.

We chose to use immunohistochemistry to examine alterations in the abundance of selected signals that might explain changes in intestinal sugar uptake. Based on a review of the literature on signals regulating intestinal sugar uptake we chose to examine eight possible signals (PKA, PKC, ERK1/2, p38, Akt, mTOR, GSK-3 and eNOS). It has been reported that PKA activation enhances both intestinal glucose (Hirsch et al., 1996) and fructose transport (Cui et al., 2003). There are conflicting reports of the effect of PKC on glucose transport (Hirsch et al., 1996; Vayro et al., 1999; Vehyl et al., 2003; Millar et al., 2002), with decreases in sugar transport observed with rabbit and rat SGLT1, and increases seen with human SGLT1 expressed in Xenopus Oocytes (Hirsch et al., 1996). PKCβII may be involved in the enhancement of glucose absorption, which is accomplished by the trafficking of GLUT2 to the BBM (Helliwell et al., 2000a; Helliwell et al., 2003), and PI3K may be important in the stimulation of sugar absorption following an oral sugar load (Helliwell et al., 2000a; Helliwell et al., 2003). The MAPK pathway has been implicated in the control of BBM fructose transport, by modulating both the levels and intrinsic activities of GLUT5 and GLUT2 (Helliwell et al., 2000a). Finally, in TPN-fed piglets GLP-2 has been shown to influence GSK-3 and eNOS (NOS3), both of which are linked to the PI3K pathway (Figure 61). These proteins may indirectly impact intestinal sugar uptake via effects on intestinal blood flow and cell proliferation (Guan et al., 2003, Burrin et al., 2005).

We recognize, of course, that cDNA array studies undertaken in animals following intestinal resection have revealed multiple other possible signals in this model of adaptation (Erwin et al., 2000; Stern et al., 2001). Therefore, by choosing only eight signals, there may have been proteins involved in the adaptive response that we did not measure. We also recognize that we are only describing associations between sugar uptake and the protein abundance of signals.

GLP-2/Dexamethasone

We first examined the changes in these signaling proteins in association with GLP-2 and DEX induced alterations in intestinal sugar uptake. We did not see significant alterations in the abundance of PKA, PKC, ERK1/2, p38, GSK-3 and eNOS. However, the changes in sugar uptake associated with GLP-2 and DEX were associated with directionally similar alterations in Akt and mTOR abundance.

The PI3K/Akt pathway is thought to transduce proliferative signals from growth factor receptors to the cell cycle machinery of intestinal epithelial cells. Indeed, Akt increases cyclin D expression, promoting entry into the S phase (Sheng et al., 2003), while mTOR regulates several cell cycle proteins including pRb and p27^{KIP1} (Asnaghi et al., 2004). Because of this link between the PI3K pathway and cellular proliferation, we also examined the effect of GLP-2 and DEX on proliferation (as assessed by PCNA staining). In most cases, alterations in Akt were accompanied by parallel changes in PCNA abundance. For example, in sucklings the decreases in ileal proliferation observed in response to DEX and GLP-2 was associated with concomitant decreases in Akt and mTOR (Figure 56). This suggests that the one of the mechanisms by which GLP-2 and DEX influenced Akt and mTOR was through initiating changes in intestinal proliferation.

Aging

Based on the results obtained from the GLP-2/DEX study we decided to examine the association between age-related changes in intestinal sugar uptake and the abundance of Akt and mTOR. The fall in sugar uptake between suckling and weanling animals was associated with parallel reductions in Akt and mTOR, but not PCNA abundance (See Appendix: Table 35). The decline in sugar uptake later in life was associated with directionally similar reductions in Akt but not mTOR or PCNA abundance (See Appendix: Table 33). This suggests that age-related changes in intestinal sugar transport are not mediated by alterations in intestinal proliferation. Still, the changes in Akt (and in some cases mTOR) indicate that this pathway is involved in a manner that does not involve transducing proliferative signals.

Dietary Lipids

Of interest, the alterations in sugar uptake associated with dietary lipids were not paralleled by similar changes in the abundance of Akt or mTOR (See Appendix: Figure 34). This suggests that the mechanism by which dietary lipids alter intestinal sugar transport and intestinal morphology occurs via an alternate mechanism that does not involve the PI3K pathway.

12.7. CONCLUSION

The characterization of intestinal sugar transport over the lifetime of the rat and the modulation of this process by GLP-2, DEX and dietary lipids increases our understanding of intestinal sugar transport. Although in some cases we have described associations between intestinal sugar uptake and the PI3K pathway, proof of this being a cause and effect relationship will only come in future studies where transporters are artificially modified using stimulators or inhibitors of these signals, and transport activity is assessed.

12.8. FUTURE STUDIES

There are many future studies that could be undertaken to further characterize the process of intestinal sugar transport in general. Several of these would be logical extensions to each of the studies that we have performed. I will first discuss these proposed future studies, and later will discuss specific suggestions for adding to information that we have obtained for the individual studies (aging, GLP-2/DEX, dietary lipids).

Sugar Transport

The mechanisms involved in the regulation of intestinal sugar transport require further characterization. The recruitment of GLUT2 to the BBM has been shown to be important under experimental conditions where high sugar perfusions or high sugar meals were given (Helliwell and Kellett, 2000; Gouyon et al., 2003) or when vascular perfusions of GLP-2 were given (Au et al., 2002). However, it is not known if this contributes significantly to sugar uptake in other models, and whether this process in influenced by age, hormones or dietary lipids. This is an important step in discovering the mechanisms by which intestinal sugar transport is regulated in health and disease.

Although there is some evidence of their existence (Cheeseman, 1997; Chung et al., (2002), Kipp et al. (2003); Au et al., 2002; Affleck et al., 2003), intracellular pools of SGLT1, GLUT2 and GLUT5 await definitive identification. Intracellular pools of other transporters have been characterized. For example, immunogold staining has been used to identify GLUT4 in intracellular pools in adipocytes (Haney et al., 1991). Quantitative confocal microscopy has been used to show that the CFTR rapidly redistributes from the cytoplasm to the apical surface upon cAMP stimulation in rat duodenum (Ameen et al., 1999). These techniques such as may be used to localize transporters to various locations within the enterocyte. The use of protein synthesis inhibitors such as cycloheximide are useful in determining whether the trafficking of transporter to the BBM requires newly synthesized protein or results from the trafficking of pre-formed protein from intracellular pools. Furthermore, blocking trafficking using specific trafficking inhibitors such as brefeldin A (inhibits translocation from trans-Golgi), cholchicine (disrupts microtubules) and cytochalasin D (inhibits actin polymerization) may enable the specific determination of where the trafficking is occurring.

Several studies have shown that alterations in sugar transport cannot by fully explained by changes in trafficking of the protein to the BBM (Cheeseman, 1997; Khoursandi et al., 2004). The relative contribution of trafficking and alterations in intrinsic activity should be determined. Alterations in intrinsic activity have been observed in several models such as with hyperglycemia (Maenz and Cheeseman, 1986), diabetes (Corpe et al., 1996), low luminal glucose concentrations (Kellett and Helliwell, 2000), and following the activation of MAPK and PI3K (Helliwell et al., 2000a). This is also true for our study. However, it is not known how the activity of the transporters are modulated. There are several possibilities For example, the transporters could be post-translationally modified by phosphorylation and glycosylation. Phosphorylation sites have been identified on SGLT1 (Wright et al., 1997) and both PKC and PKA do alter sugar transport, although there are differences in the effect depending on the species (Vayro et al., (1999); Vehyl et al., (2003), Hirsch et al., (1996). Phosphorylation sites have not been identified on GLUT2 or GLUT5, although the GLUT5 promoter does contain two cAMP response elements (Mahraoui et al., 1994).

Regulatory proteins may interact with transporters and influence transport activity. For example, SGLT1 activity is modified by RS1 (Veyhl et al., 2003; ref), and mice lacking this protein exhibit increased intestinal glucose transport and develop obesity (Osswald et al., 2005). Heat shock proteins influence SGLT1 activity in renal epithelial cells (Ikari et al., 2002). The effect of these proteins on SGLT1 in the intestine requires further study. Also, it is not known if there are similar regulatory proteins that may modify the activity of other sugar transporters such as GLUT2 and GLUT5.

A number of other factors have recently been shown to influence intestinal sugar transport. *Foxl1* is a winged-helix transcription factor expressed in the mesenchymal cells bordering the crypts in the small intestine. *Foxl1* null mice had decreased intestinal glucose uptake and decreased levels of SGLT1 protein (Katz et al., 2004). Furthermore, recent studies have demonstrated that AMP-activated kinase (AMPK), an intracellular energy sensor, resulted in the recruitment of GLUT2 to the BBM and a down-regulation of the energy-requiring SGLT1-mediated glucose uptake (Walker et al., 2004). The importance of these factors, particularly in models of intestinal damage or stress, warrants further investigation.

It has previously been shown that dietary lipids alter membrane lipid composition (Keelan et al., 1990). This may result in altered transport due to changes in the lipid environment near the transporter, which may influence the configuration and binding sites. In renal proximal tubular cells, SGLT1 was found in detergent-resistant membrane microdomains, also referred to as lipid rafts (Runembert et al., 2002). Similarly, GLUT1 has been localized to lipid rafts in adipocytes (Kumar et al., 2004). Although preliminary studies have not been able to identify sugar transporters in lipid rafts of the intestine (Park et al., unpublished observations, 2005), further research is required to determine if SGLT1 is localized to lipid rafts in the intestinal BBM, if this localization is mandatory for the functioning of SGLT1, and what factors may regulate the localization of SGLT1 to these specialized microdomains. Of interest, PI3K and Akt have been localized to lipid rafts of the intestine (Li et al., 2004). The possible relationship between PI3K and sugar transporters in lipid rafts should be explored.

Although our studies did not show significant changes in the distribution of transporter protein, the mechanisms responsible for the activation of proteins along the

CV axis remains an interesting area of study. Akt abundance along the CV axis is variable with increased expression of Akt1, and decreased expression of Akt2, during enterocytic differentiation (Dufour et al., 2004). While this expression pattern may simply reflect the roles of these proteins in cell survival and cell death, it could be interesting to correlate the expression of these proteins with the expression of transport activity along the CV axis using phloridzin binding studies.

Signals

Future studies should be aimed at further characterizing the role of the PI3K pathway in the regulation of intestinal sugar transport using inhibitors of this pathway (ie. wortmannin, LY294002). By no means does the directionally similar association of changes in Akt and sugar uptake prove a cause and effect relationship. It would be important to know whether this relationship existed, because it would provide a potential new approach to the modulation of glucose uptake. This could be potentially useful, for example, to decrease sugar uptake under clinical situations where this was desirable, such as in obesity and diabetes, or to increase uptake under conditions of malabsorption.

If inhibitor studies do show that Akt does alter transport, it would be interesting to determine if the mechanism primarily involves alterations in trafficking or intrinsic activity. For example, in adipose and muscle PI3K alters GLUT4 trafficking, while MAPK alters GLUT4 activity (Harmon et al., 2004). It is not known if this is the case in the intestine, as Helliwell et al. (2000) have suggested that there is extensive cross-talk between the PI3K and MAPK pathways in the regulation of GLUT2 and GLUT5 activity. Future studies using stimulators and inhibitors of these pathways, as well as inhibitors of the transporters may further elucidate the specific pathways responsible for regulating the intracellular trafficking and activity of intestinal sugar transporters.

In our studies, we have shown an association between Akt and PCNA. This raises the issue whether these are both activated by the same stimulus, or if one changes secondary to the other. This could be determined by increasing or decreasing Akt and following the time course of changes in PCNA or *vice versa*.

We have examined the role of selected signals in the regulation of sugar transport. Alternatively, cDNA or proteomic technology could be applied to the three models used (aging, GLP-2/ DEX, and dietary lipids) to discover novel signals.

FUTURE STUDIES: AGING

The data shows that adaptation results observed in one age group cannot be applied to another group. Indeed, feeding SFA to old animals decreases not increases uptake. This suggests that the use of PUFA may have unwanted metabolic consequences because of the anticipated and paradoxical way in which the old intestine adapts. Diet studies done specifically on aged animals or elderly humans should be done to determine the effects of nutrients in these populations.

It would be interesting to test the hypothesis of Ferraris' group that suggests that the suckling intestine may be incapable of adaptation due to the constant composition of milk (Jiang et al., 2001). This idea is intriguing, however it has been shown that even the fetal intestine can adapt to changes in the sugar composition of the amniotic fluid (Buchmiller et al., 1992; Dyer et al., 1992). The ability of the intestine to adapt its transport capabilities at various stages of development could be studied in the future.

It would be of interest to further test if the age-related changes in intestinal sugar transport are indeed "hard-wired" or whether they are influenced by environmental factors.

The decline in intestinal Akt with age observed in our study and others (Gentili et al., 2003) begs the question: Does Akt decline in other tissues with age? Indeed there is evidence of reductions in Akt in the pancreas (Watanabe et al., 2005), kidney (Parekh et al., 2004) and fibroblasts (Lorenzini et al., 2002). The role of the PI3K pathway in age-related changes in organ function should be investigated.

Finally, the role of other age-related genes in the regulation of intestinal function should be further studied. For example, a pilot study done in cooperation with Dr. Halloran's group in the Division of Nephrology showed age-related increases in the intestinal mRNA expression of $p16^{INK4a}$ (See Appendix: Figure 64). This gene is a marker of chronological age in the kidney, and may be an indicator of premature senescence caused by stresses or disease. The role of this gene and other similar genes in the age-associated changes in intestinal function is of interest.

PROGRAMMING

In our studies the administration of GLP-2 and DEX during suckling or during lactations produced few significant effects on intestinal sugar transport in suckling and weanling animals. However, when these peptides were given during pregnancy, significant changes were observed. Therefore, the results of these studies suggest that the prenatal period may be important for the programming of intestinal function. Further studies should be conducted to determine if this is indeed the case. If so, the importance of prenatal nutrition and other prenatal factors, such as the use of steroids, should be emphasized.

It is recognized that events that occur early in life may have a lasting effect into adulthood (Karasov et al., 1985; Barker et al., 1993; Seckl, 1998). In our studies the animals were followed for four weeks after the administration of GLP-2 and DEX. It would be important to have a longer follow-up period to determine the long-term effects of these agents on intestinal function and nutritional status.

FUTURE STUDIES: GLP-2/DEX

Although maternal GLP-2 did influence intestinal transport in offspring we did not determine if this effect was direct or indirect. Does GLP-2 cross the placenta? Does GLP-2 pass into breast milk? These two questions must be answered in order to understand the mechanism of action of this hormone.

Recent studies have shown that there may be interactions between GLP-2 and other hormones/growth factors, such as EGF. For example, Kitchen et al. (2005) showed that intestinal growth in parenterally fed rats was increased to a further extent when GLP-2 and EGF were combined, when compared to either agent given alone. Furthermore, GLP-2 may be exerting an indirect effect via stimulation of other factors, such as KGF (Orskov et al., 2005). The relationship between GLP-2 and other factors should be studied further. This may lead to the discovery of an optimal combination of factors for potential clinical use in situations such as short bowel syndrome where the stimulation of intestinal transport may be desirable.

Also, while the gut has traditionally been thought of as an insulin-insensitive tissue, studies have suggested that the rat small intestine is insulin-sensitive (Croset et al., 2001). Furthermore, oral insulin has trophic effects on the small intestine (Shamir et al., 2005) and normalizes intestinal sugar transport in diabetic animals (Thomson and Rajotte, 1985). It would be important to determine if the effects of GLP-2 and particularly DEX are related to insulin. More research into the role of insulin in the regulation of gut function is warranted.

FUTURE STUDIES: DIETARY LIPIDS

In our study, dietary lipids influenced morphology and transport. These changes were not associated with alterations in PCNA, Akt or mTOR. Thus, the mechanism of action of dietary lipids has not been elucidated. Previous studies have shown parallel alterations in c-jun, ODC and proglucagon with steroids and dietary lipids following intestinal resection (Thiesen et al., 2003c). More studies are required to fully characterize the mechanism by which dietary lipids influence transport. Whether the effect is a direct effect on the enterocyte, or an indirect effect due to hormone secretion should be explored.

12.9. FIGURES

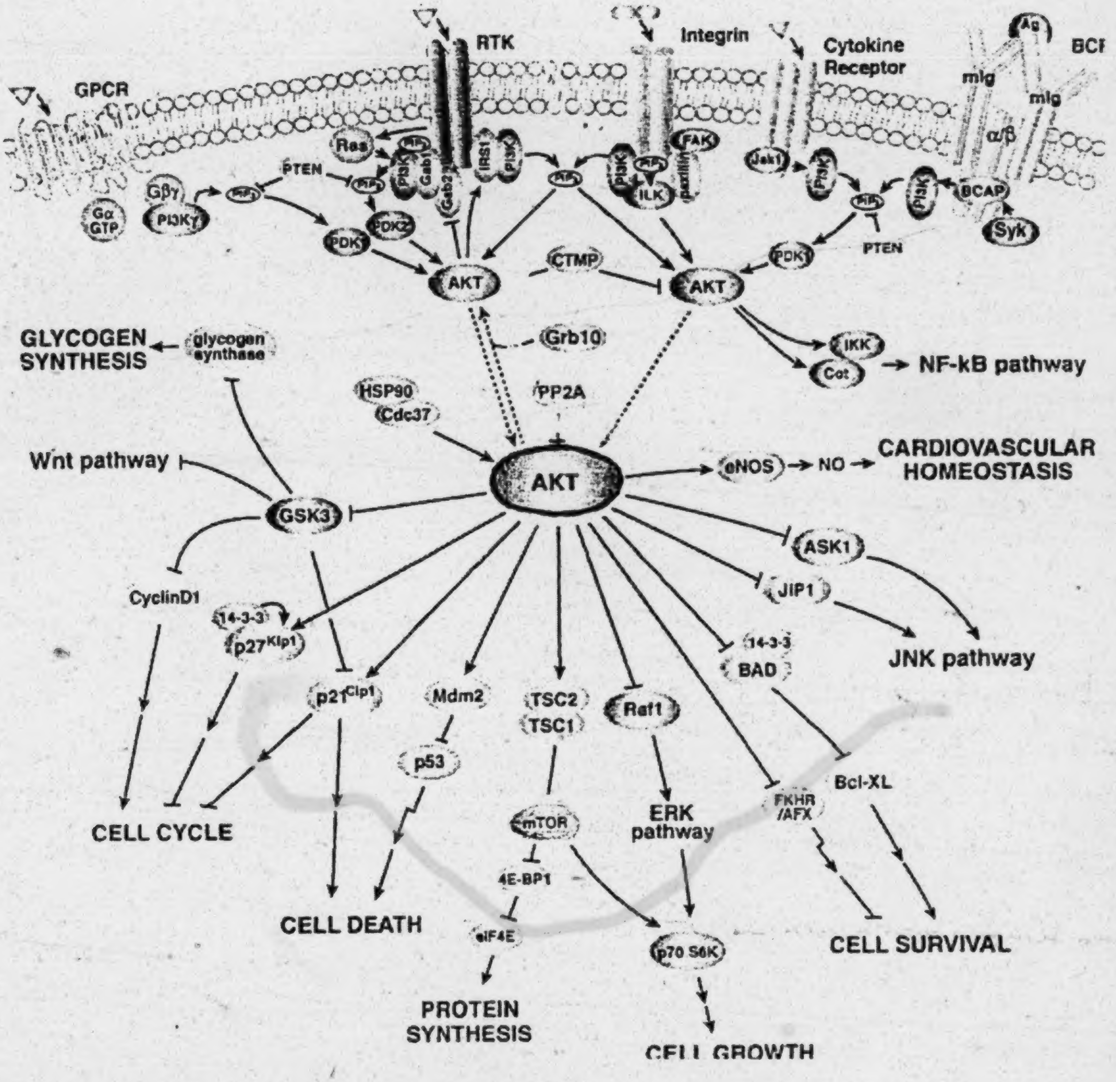
GPCR
RTK
Integrin
Cytokine Receptor
Ag
BCI
mIg
mIg
α/β
Gα GTP
Gβγ
PI3Kγ
PTEN
Ras
PI3K
Gab1
Gab2
IRS1
PI3K
FAK
PI3K
ILK
paxillin
Jak1
PI3K
PI3K
BCAP
Syk
PDK2
PDK1
PDK1
PTEN
AKT
CTMP
AKT
Grb10
IKK
Cot
NF-kB pathway
GLYCOGEN SYNTHESIS
glycogen synthase
HSP90
Cdc37
PP2A
AKT
eNOS
NO
CARDIOVASCULAR HOMEOSTASIS
Wnt pathway
GSK3
ASK1
JIP1
JNK pathway
CyclinD1
14-3-3
p27Kip1
p21Cip1
Mdm2
TSC2
TSC1
Raf1
14-3-3
BAD
p53
mTOR
ERK pathway
FKHR /AFX
Bcl-XL
CELL CYCLE
4E-BP1
CELL DEATH
eIF4E
p70 S6K
CELL SURVIVAL
PROTEIN SYNTHESIS
CELL GROWTH

Figure 61. The PI3-kinase/Akt Pathway (from CellSignal.com)

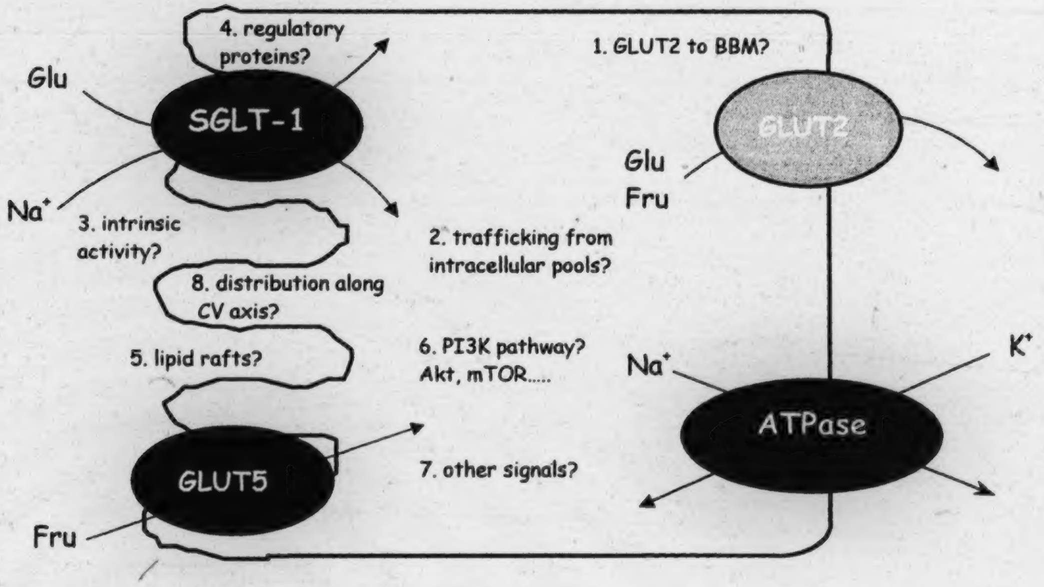
4. regulatory proteins?
1. GLUT2 to BBM?
Glu
SGLT-1
GLUT2
Glu
Fru
Na$^+$
3. intrinsic activity?
2. trafficking from intracellular pools?
8. distribution along CV axis?
5. lipid rafts?
6. PI3K pathway? Akt, mTOR.....
Na$^+$
K$^+$
ATPase
GLUT5
7. other signals?
Fru

Figure 62. Possible alternate mechanisms involved in the regulation of intestinal sugar transport

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13. APPENDIX

Table 33. The effect of age on jejunal Akt and mTOR protein abundance in 1, 9 and 24 month old rats

		CHOW				**SFA**				**PUFA**			
		mean		**SEM**		**mean**		**SEM**		**mean**		**SEM**	
Akt	**1 month old**	64.9	±	6.7	**a**	59.4	±	5.1	**a**	42.7	±	5.8	**a**
	9 months old	73.4	±	3.7	**a**	66.7	±	5.7	**a**	68.5	±	3.6	**b**
	24 months old	43.6	±	6.5	**b**	73.9	±	5.0	**a**	70.5	±	4.4	**b**
mTOR	**1 month old**	55.1	±	7.3	**a**	72.7	±	4.8	**a**	65.4	±	9.0	**a**
	9 months old	67.7	±	6.6	**a**	71.9	±	11.3	**a**	71.1	±	7.8	**a**
	24 months old	71.2	±	4.1	**a**	73.4	±	6.7	**a**	57.3	±	8.0	**a**
PCNA	**1 month old**	74.6	±	5.5	**a**	63.2	±	10.6	**a**	51.5	±	6.9	**a**
	9 months old	64.6	±	2.3	**a**	54.4	±	12.3	**a**	56.5	±	5.3	**a**
	24 months old	67.7	±	6.1	**a**	68.1	±	8.8	**a**	63.5	±	3.2	**a**

Mean ± SEM, n=4
Age effect: means with different letters are significantly different ($p \leq 0.05$)
No significant diet effect (PUFA vs. SFA)

Table 34. The effect of a PUFA and SFA diet on ileal Akt protein abundance in 1,9 and 24 month old rats

	1 month old								9 months old								24 months old						
	SFA				PUFA				SFA				PUFA				SFA				PUFA		
	mean		sem		mean		sem		mean		sem		mean		sem		mean		sem		mean		sem
Akt	13.0	±	3.4		19.2	±	4.4		15.7	±	2.2		17.8	±	3.4		23.4	±	2.7		16.7	±	2.9
MTOR	26.4	±	1.2		23.0	±	2.0		24.0	±	3.4		23.4	±	2.3		20.1	±	1.5		27.3	±	4.2

Mean ± SEM, n=4-5
No statistically significant differences.

Table 35. Comparisons between the jejunal parameters of control suckling and weanling animals

	Sucklings				Weanlings			
JEJUNUM	**mean**		**sem**		**mean**		**sem**	
Villous height	270	±	35		149	±	49	
Villous width (base)	72	±	2		46	±	14	
Villous width (mid)	64	±	3		53	±	14	
Crypt depth	48	±	4		63	±	12	
Villous density	509	±	39		255	±	64	*
Enterocyte Size	30	±	0.5		11	±	3	*
Fructose uptake	32.7	±	1.8		7.0	±	1.0	*
Glucose Uptake (Vmax)	3710	±	322		1624	±	316	*
Glucose Uptake (Km)	21	±	4		22.2	±	10.2	
PCNA	35.1	±	2.9		55.6	±	2.7	*
Akt	50.7	±	2.5		35.0	±	0.9	*
MTOR	43.2	±	1.5		36.7	±	3.6	
SGLT1	42.1	±	8.4		38.3	±	5.9	
GLUT2	53.1	±	4.2		55.1	±	12.0	
GLUT5	35.7	±	6.9		64.3	±	3.3	*
	Sucklings				**Weanlings**			
	mean		**sem**		**mean**		**sem**	
ILEUM								
Villous height	219	±	10		288	±	44	
Villous width (base)	57	±	3		117	±	14	*
Villous width (mid)	58	±	2		113	±	21	*
Crypt depth	33	±	3		88	±	12	*
Villous density	425	±	16		893	±	170	*
Enterocyte Size	27	±	2		42	±	7	
Fructose uptake	23.3	±	3.2		12.5	±	0.8	*
Glucose Uptake (Vmax)	4805	±	1559		1050	±	184	*
Glucose Uptake (Km)	41.8	±	26		13.9	±	6.8	
PCNA	68.4	±	1.2		70.6	±	1.5	
Akt	55.1	±	0.7		46.4	±	0.4	*
MTOR	55.4	±	1.2		32.5	±	3.4	*
SGLT1	54.2	±	3.2		41.3	±	3.1	*
GLUT2	70.1	±	2.2		42.8	±	4.4	*
GLUT5	48.9	±	3.3		51.9	±	9.8	

-data taken from control animals in the Pregnancy study (Chapters 10 and 11)

-t-tests performed to determine statistical significance ($p \leq 0.05$)

Table 36. Microvillous morphology in 1, 9 and 24 month old rats

	1 m		9 m		24 m	
JEJUNUM	**mean**	**sem**	**mean**	**sem**	**mean**	**sem**
Microvillous height (um)						
CHOW	1.04	± 0.03	1.45	± 0.05	0.75	± 0.01
PUFA	NA		NA		1.11	± 0.04
SFA	NA		NA		0.96	± 0.03
Microvillous width (um)						
CHOW	0.13	± 0.00	0.10	± 0.00	0.10	± 0.00
PUFA	NA		NA		0.12	± 0.00
SFA	NA		NA		0.11	± 0.00
# microvilli/1.5um						
CHOW	12.00	± 1.00	11.00	± 1.00	13.67	± 0.33
PUFA	NA		NA		12.00	± 0.58
SFA	NA		NA		11.33	± 0.67

	1 m		9 m		24 m	
ILEUM	**mean**	**sem**	**mean**	**sem**	**mean**	**sem**
Microvillous height (um)						
CHOW	0.92	± 0.01	1.29	± 0.02	0.83	± 0.06
PUFA	NA		NA		0.87	± 0.02
SFA	NA		NA		1.60	± 0.08
Microvillous width (um)						
CHOW	0.12	± 0.00	0.11	± 0.00	0.13	± 0.00
PUFA	NA		NA		0.11	± 0.00
SFA	NA		NA		0.10	± 0.00
# microvilli/1.5um						
CHOW	12.67	± 0.88	13.67	± 0.67	10.50	± 0.65
PUFA	NA		NA		12.25	± 0.95
SFA	NA		NA		13.00	± 0.71

Mean ±SEM, n=1
Samples were fixed in glutaraldehyde/osmium tetroxide.
Measurements determined using Transmittance Electron Microscopy
Pictures were taken from the mid-villous region
3 pictures per sample, 10 measurements per picture
NA: no measurements were taken for these samples

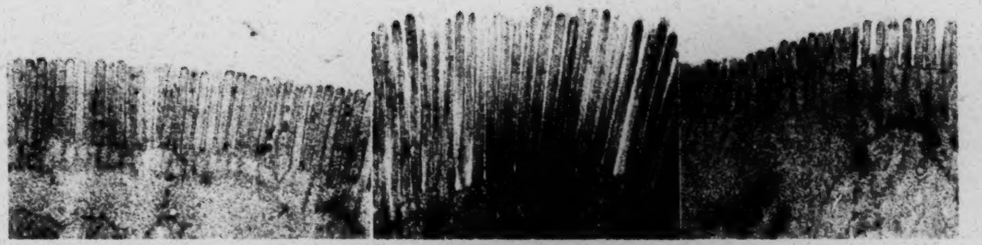
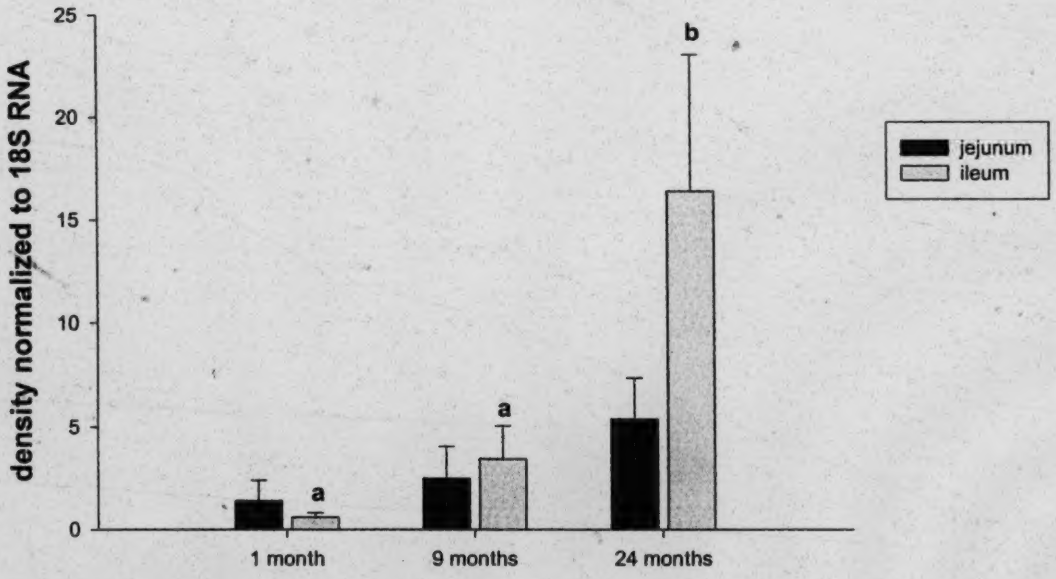


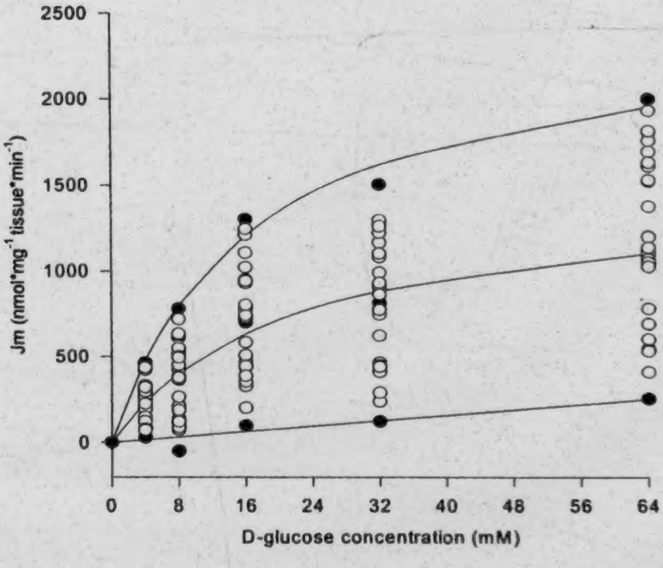
Figure 63. Representative Transmittance Electron Microscopy images from a) 1, b) 9 and c) 24 month old chow-fed rats.

Figure 64. p16^{INK4a} mRNA expression in 1, 9 and 24 month old rats



Mean±SEM, n=4
Total RNA was extracted from tissue samples according to a modification of the method described in Chirgwin et al. (1979). Transcription into cDNA was done using MMLV reverse transcriptase and random primers (Life Biotechnologies, Burlington, ON). DNA was then amplified in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Sequence specific primers and probe for p16^{INK4a} were designed using Primer Express software (Applied Biosystems, Foster City, CA). Values were normalized to 18S RNA. An internal control was run with each experiment.

Figure 65. Sample glucose and fructose plots



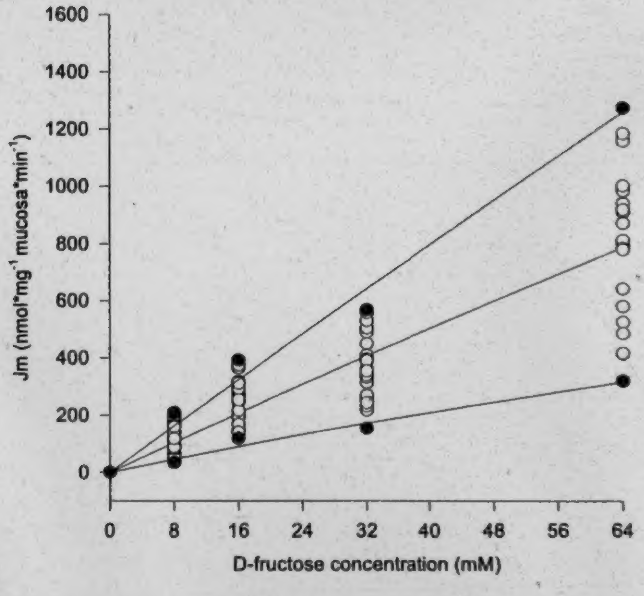


Figure 66. Sample linear transformations